

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
31 July 2003 (31.07.2003)

PCT

(10) International Publication Number
WO 03/062279 A1

(51) International Patent Classification⁷: C08B 37/00,
C07K 14/78, A61K 31/737, 31/726, 38/39, A61P 19/04,
29/00, C12S 3/02, 3/14

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(21) International Application Number: PCT/AU03/00061

(22) International Filing Date: 23 January 2003 (23.01.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PS 0112 23 January 2002 (23.01.2002) AU
PS 1054 12 March 2002 (12.03.2002) AU

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(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

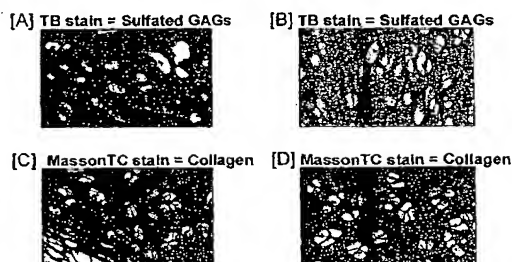
— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NUTRACEUTICALS FOR THE TREATMENT, PROTECTION AND RESTORATION OF CONNECTIVE TISSUES

Bovine Tracheal Cartilage

- before [A & C] and after treatment [B & D]



(57) Abstract: The invention relates to a method for isolating from connective tissue a variety of glycosaminoglycan (GAG)-peptide complexes and polypeptides which are substantially free of contaminating DNA and other molecules such as viruses which may be associated with the DNA in the cell. The invention also relates to uses of GAG-peptide complexes and polypeptides substantially free of DNA either directly, or after further processing, for the treatment, protection and restoration of connective tissues in inflammatory and degenerative disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms or other degenerative conditions in mammal. Figure 2 shows photomicrographs (magnification X 400) of histological sections of 3mm bovine tracheal cartilage powders particles before and after subjecting them to the autolysis procedure using 100mM calcium acetate buffer, pH 4.5 at 37 degrees C. Panels A and C are sections of cartilage before autolytic processing and B and D after. Panels A and B show the results of staining with Toluidine Blue (TB), a dye which binds to glycosaminoglycans (GAGs) while C and D show sections stained with Masson Trichrome (Masson TC), a dye known to stain native collagen fibres magenta colour. Note the loss of staining for GAGs in section B after autolysis but a slightly increased intensity of staining for collagen following the removal of the majority of GAGs as shown in D. Cell nuclei, identified in these sections by the green counterstains are clearly unchanged by the autolytic process.

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NUTRACEUTICALS FOR THE TREATMENT, PROTECTION AND
RESTORATION OF CONNECTIVE TISSUES

Technical Field

- 5 The invention relates to a method for isolating from connective tissue a variety of glycosaminoglycan (GAG)-peptide complexes and polypeptides which are substantially free of contaminating DNA and other molecules such as viruses which may be associated with the DNA in the cell. The invention also relates to uses of GAG-peptide complexes and polypeptides substantially free of DNA either directly, or
10 after further processing, for the treatment, protection and restoration of connective tissues in inflammatory and degenerative disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms or other degenerative conditions in mammals without potential contamination with viruses and other pathogens which are known to be localised within cells and bound to some of their intracellular components.
- 15 The invention is also directed to the use of a mixture of the Calcium salt of a GAG-peptide complex(es) and polypeptides, preferably substantially free of DNA, which has been found to enhance connective tissue cell anabolism and macromolecular biosynthesis as well as exhibit immuno-suppressive and anti-inflammatory activities in an animal model. By improving connective tissue cell proliferation, matrix gene
20 expression and inflammation by the above mentioned preparations, even in the presence proinflammatory mediators, such as Interleukin-1 which are known to suppress cellular biosynthesis of matrix components and promote their degradation, these naturally derived GAG-peptide complexes and polypeptides can slow or halt the progression of tissue destruction and inflammation which are characteristic
25 pathological features of arthritis and related degenerative diseases.

Background Art

- Musculoskeletal disorders such as rheumatoid arthritis (RA), osteoarthritis (OA), disc degeneration (DD) and osteoporosis (OP) are the major cause of morbidity throughout the world. These diseases have a substantial influence on health and quality
30 of life and inflict an enormous cost on health systems (Scott JC, Hochberg MC. Arthritic and other musculoskeletal diseases, In: Chronic Disease Epidemiology, Brownson RC, Remington PL and Davis JL, eds. Washington, DC. American Public Health Association, 1993). It has been estimated that musculoskeletal diseases cost the Australian community \$1.3 billion annually in direct costs and \$4.2 billion in indirect
35 costs. This represents 1.3% of GNP (Arthritis Foundation of Australia, Access Economics Pty Ltd report, 2001). Population based studies conducted in other

developed countries show similar incidence and health burdens to those of Australia with for example more than 23 million Americans seeking medical treatment for arthritic disorders with costs associated with their management exceeding 70 billion dollars annually (Ruchlin HS, Elkin EB, Paget SA. Assessing cost-effectiveness analyses in rheumatoid arthritis and osteoarthritis. *Arthritis C Res* 1997;10:413-21). Moreover, the prevalence of OA, DD and OP is rising progressively as the life span of the peoples of developed nations increases. Indeed, it has been estimated that 20% of the population in developed countries will be actively seeking medical treatments or professional health support for musculoskeletal disabilities by the year 2020 (Croft P. The occurrence of osteoarthritis outside Europe. *Ann Rheum Dis* 1996;55:661-4).

The aetiology of musculoskeletal conditions such as OA is multifactorial and although ageing is the most strongly associated risk factor, mechanical, hormonal and genetic factors all contribute to varying degrees. OA emerges as a clinical syndrome when these etiological determinants result in sufficient joint damage to cause impairment of function and the appearance of symptoms. This clinical syndrome is manifest radiologically by joint space narrowing due to loss of articular cartilage (AC) and extensive re-modelling of subchondral bone with proliferation at the joint margins (osteophytosis). In the late stages of OA, joints are characterised pathologically by extensive AC fibrillation, loss of staining for proteoglycans (PGs) and eburnation of bone at sites of high contact stress. The subchondral bone beneath those regions of OA where cartilage is fibrillated or eroded is generally sclerotic and consists of immature woven bone (Felson DT. Osteoarthritis. *Rheum Dis Clin Nth Amer* 1990;16:499-512).

The PGs of AC consist of a protein core to which several hundred GAG chains are covalently attached. The major GAG substituents of the PGs of AC are the Chondroitin sulfates (ChS) whose isomeric structures and their distribution are well known (Poole AR. Changes in collagen and proteoglycan of articular cartilage in arthritis. *Rheumatology*, 1986, 10; 316-371). Thus in adult cartilage Ch-6-S is more abundant than the corresponding 4-sulfated isomer (Ch-4-S) which predominates in AC of very young animals. Within the extracellular matrix of AC the hydrated PG complexes are entrapped in the form of macromolecular aggregates by a three dimensional network of Type II collagen fibres. This unique structural organisation of PGs, water and a fibrous collagen network which is anchored in the subchondral bone plate confers to AC the biomechanical properties of resilience necessary for normal biomechanical function (Poole AR. Changes in collagen and proteoglycan of articular cartilage in arthritis. *Rheumatology*, 1986, 10; 316-371).

Products of cartilage breakdown in OA and RA joints have been shown to be antigenic [Glant TT, Fülöp C, Cs-Szabó G, Buzas E, Ragasa D, Mikecz K. Mapping of arthritogenic/autoimmune epitopes of cartilage aggrecans in proteoglycan-induced arthritis. *Scand J Rheumatol* 1995;24:43-9, Rowley M, Tait B, Mackay IR, Cunningham T, Phillips B. Collagen antibodies in rheumatoid arthritis: Significant of antibodies to denatured collagen and their association with HLA-DR4. *Arthritis Rheum* 1986;29:174-84, Seibel MJ, Jelsma R, Saed-Nejad F, Ratcliffe A. Variability in the immunochemical quantification of keratan sulfate in human and bovine cartilage proteoglycans. *Biochem Soc Trans* 1990;18(5):969-70] and when released into synovial fluid (SF) may provoke a synovial inflammation. This synovitis, once established, can alter the metabolism of resident synoviocytes, the major cellular source of synovial hyaluronan (HA) in joints. Inflammatory mediators released from local macrophage and infiltrating leukocytes can also promote increased vascular permeability and the dilution of SF by plasma fluid, thereby decreasing local HA concentration (Müller-Ladner U, Gay RE, Gay S. Structure and function of synoviocytes. In: *Arthritis and Allied Conditions*, Koopman WJ, ed. Baltimore. Williams and Wilkins, 1997, 243). This dilution of HA coupled with a reduction in its molecular size due to abnormal synthesis by synoviocytes results in a substantial decrease in the rheological properties of SF and consequently its ability to lubricate and protect AC (Balazs EA. The physical properties of synovial fluid and the special role of hyaluronic acid. In: *Disorders of the Knee*, Helfet AJ, ed. Philadelphia. JP Lippincott, 1982, 61-74). Macrophage of the synovium, together with the leukocytes which enter the synovial cavity due to the local inflammation, are also an abundant source of cytokines [eg interleukin-1 (IL-1)], procoagulant factors, proteinases and oxygen-derived free radicals including nitric oxide radical (NO) (Pelletier J-P, DiBattista JA, Roughley P, McCollum R, Martel-Pelletier J. Cytokines and inflammation in cartilage degradation. *Rheum Dis Clin N Am* 1993;19:545-68, Dean DD. Proteinase-mediated cartilage degradation in osteoarthritis. *Sem Arthritis Rheum* 1991;20:2-11). While much of the excess proteolytic activity released into synovial fluid is abrogated by the endogenous inhibitors present, cytokines and free radicals can freely diffuse into cartilage and down-regulate PG and collagen synthesis by chondrocytes. These proinflammatory mediators can also initiate the production of catabolic proteinases, cytokines and free radicals such as NO - by the cartilage cells which via autocrine and paracrine pathways contribute to further AC matrix destruction (Evans CH, Watkins SC, Stefanovic-Racic M. Nitric oxide and cartilage metabolism. *Methods Enzymol* 1996;269:75-88).

It is clear from the above that in arthritic diseases such as OA all tissues of the joint are affected and their excessive breakdown and the concomitant elicitation of an inflammatory reaction can lead not only to the progression of the disease state but also the initiation of symptoms the most common being pain and impairment of joint function.

Pharmacological management of rheumatic disorders and back pain of discal origin, has up until quite recently, targeted the symptoms of these diseases rather than the underlying pathologies which are the cause of the symptoms. Analgesics, steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) have over the last 50 years, represented the mainstay of pharmacological treatment for the rheumatic diseases. However, the deleterious side effects associated with the use of many of these synthetic drugs (Lichtenstein DR, Syngal S, Wolfe MM. Nonsteroidal antiinflammatory drugs and the gastrointestinal tract. The double-edged sword. *Arthritis Rheum* 1995;38:5-18, Davies MN, Wallace JL. Nonsteroidal anti-inflammatory drug-induced gastrointestinal toxicity. New insights into an old problem. *J Gastroenterol* 1997;32:127-33, Manoukian AV, Carson JL. Nonsteroidal anti-inflammatory drug-induced hepatic disorders. Incidence and prevention. *Drug Safety* 1996;15:64-71, Huskisson EC, Berry H, Gishen P, Jubb RW, Whitehead J. Effects of antiinflammatory drugs on the progression of osteoarthritis of the knee. *J Rheumatol* 1995;22:1941-6) has prompted the evaluation and development of alternative treatments, particularly remedies from edible plant and animal sources which, by their very nature, are expected to be free of adverse side-effects.

The most widely used products in this regard are glucosamine and chondroitin sulphate (ChS) which are both constituents of cartilage PGs. Although it should be noted that the glucosamine used commercially is generally isolated from the chitosan present in the exoskeleton of crustacea. Controlled clinical studies conducted with glucosamine and chondroitin sulfate, alone or in combination, have indicated that they can provide relief of symptoms in OA (McAlindon TE, LaValley MP, Gulin JP, Felson DT, Glucosamine and Chondroitin for the treatment of Osteoarthritis: a systematic quality assessment and meta-analysis. *JAMA*, 2000; 263: 1469-1475). These agents have been categorised as slow acting disease modifying anti-osteoarthritis nutraceuticals. We are also aware of a number of patent disclosures describing the use of these agents alone and in combination with various other medicants for the treatment of OA and other musculoskeletal disorders (US Patent 5,364,845, Nov 15 1994; US Patent 6,136,795, Oct 24 2000; US Patent 6,162,787, December 19, 2000, US Patent

6,271,213, Aug 7, 2001, US Patent 6,432,929, August 13 2002, and references cited therein).

Since the ChSs are obtained from natural sources they can be sold directly to the public as food additives or supplements and are not presently required to comply with the rigorous quality control criteria used for synthetically manufactured pharmaceuticals as required by government agencies such as the FDA. Commercially available chondroitin sulfates are normally manufactured from bovine tissues such as lung and trachea by hydrolysis of the GAG protein core linkage of the cartilage PGs using either chemical or enzymatic procedures (US Patent , 1,950,100 Mar 1932, Australian Patents AU-A1-66307/80 Jan 1981, AU-A-70540/87 Dec 1987, US Patent 6,162,787, December 19, 2000 and references cited therein). The negatively charged water soluble ChS may be separated and purified from the proteins and peptides also generated by the hydrolysis of cartilage by multiple precipitations with acetone, aliphatic alcohols or the formation of water insoluble complexes with quaternary ammonium salts such as cetyl pyridinium chloride (CPC) (US Patent , 1,950,100 Mar 1932, Australian Patents AU-A1-66307/80 Jan 1981, AU-A-70540/87 Dec 1987). However, none of these methods readily remove the contaminating nucleic acids (DNA and RNA) and other intracellular components also released during the chemical or enzymatic disruption of cartilage since these macromolecules are also anionic and would co-precipitate with the anionically charged ChS.

Contaminating nucleic acids could be selectively removed from the ChS by digestion with enzymes which degrade these contaminating molecules (eg, ribonucleases such as Benzonase (Merck)), however this is an expensive procedure and the enzymes used would still have to be removed from the ChS preparation at some stage. Chaotropic solvents such as guanidine hydrochloride and salt solutions of high ionic strength, such as potassium chloride have also been previously used to extract native proteoglycans from cartilaginous tissues but at low temperatures (below 4 degrees C) and with the addition of protease inhibitors to prevent degradation of the required macromolecules by endogenous enzymes (Hascall VC and Sajdera SW, Protein-polysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. J.Biological Chem. 1969,244;2384-2396; Oegema T, Hascall V, Dziwiatkowski D, Isolation and characterisation of proteoglycans from the rat chondrosarcoma. J. Biol. Chem. 1975, 250: 6151-6159; Inerot S and Heinegard D, Bovine tracheal cartilage proteoglycans. Variations in structure and composition with age. Collagen and Related Research, 1983, 3: 245-262). However, in order to release the GAG-peptides or ChS chains from these so

isolated proteoglycan complexes it is necessary to subject them to proteolytic digestion by the addition of exogenous enzymes such as papain to degrade the their protein core(US Patent 6,162,787, December 19, 2000, Inerot S and Heinegard D, Bovine tracheal cartilage proteoglycans. Variations in structure and composition with age. Collagen and Related Research, 1983, 3: 245-262, and references cited therein). Furthermore, use of these high ionic strength or chaotropic conditions to extract cartilage also disrupts cell membranes and thus release intra-cellular components, such as nucleic acids into the aqueous medium along with the PGs.

Uncharacterised GAG-peptides have also been prepared from bovine nasal cartilage but not bovine tracheal cartilage using sodium acetate or water at pH 4.5 (Nakano T, Nakano K, Sim JS, Extraction of glycosaminoglycan peptide from bovine nasal cartilage with 0.1 M sodium acetate, J Agriculture and Food Chemistry, 1998, 46; 772-778, Nakano T, Ikawa N, Ozimek L, An economical method for the extraction of chondroitin sulfate-peptide from bovine nasal cartilage. Can Agric Engineering, 2000, 42; 205 -208). However, neither the presence or absence of DNA in these GAG-peptide preparations nor their respective pharmacological activities nor direct use for the treatment of musculoskeletal conditions described could be inferred from the information recorded in these publications. Again, in the Nakano *et al* disclosures subsequent proteolytic or chemical hydrolysis of the products isolated by this method of extraction was necessary to obtain the ChS ultimately required by the authors.

In this regard it is important to note that most of the proteolytic enzymes used to exhaustively digest connective tissues to manufacture products of commercial interest, such as the ChSs are derived from bacterial or plant sources because of their broad range of substrate specificity and widespread availability. The amino acid sequences which are recognised and cleaved by these enzymes, as well as the amino acid sequences of the polypeptide fragments generated by their proteolytic actions, are therefore different to the sites of cleavage and polypeptide sequences produced by the endogenous proteinases of mammalian connective tissues. For example, studies with the mammalian class of cysteine proteinases, the Cathepsins, have shown that their preferred substrate binding and catalytic cleavage sites are different from that of the plant derived cysteine proteinase, papain (Barrett Aj, Buttle DJ, Mason Rw, Lysosomal cysteine proteinases, ISI Atlas of Science, 1988: 256 - 260). In addition, while digestion of purified preparations of PGs with papain released single ChS chains with about 10 amino acid stubs still glycosidically attached the corresponding digestions of cartilage PGs with the cathepsins, D or B or G produced clusters containing 2 or more ChS chains and longer amino acid stubs with amino acid sequences different to those

generated by the papain digested PGs (Roughley PJ and Barrett AJ, The degradation of cartilage proteoglycans by tissue proteinases, *Biochem J*, 1977, 167: 629-637).

Certain patent disclosures cite methods of preparation and use of hydrolysates of cartilage for the treatment of musculoskeletal disorders and joint cartilage defects but these inventions are limited to the use of the peptides produced from type I and type II collagens for such treatments and make no reference to pharmacological activities of any GAG-peptide complexes when used alone or in combination with these collagen derived polypeptides. Furthermore none of these previous disclosures recognises the absence or presence of intra-cellular contaminants such as nucleic acids in their preparations (US Patent 3,966,908, June 29, 1976, US Patent 4,804,745 Feb 1989, US Patent 5,399,347 Mar 1995, US Patent 5,364,845 Dec 1996, US Patent 6,025,327 Feb 2000, US Patent 6,372,794 April 2002).

While the consequences of long term human consumption of bovine or other animal nucleic acids in commercial ChS preparations sold as nutraceuticals or food supplements is presently unknown, it should be noted that these intracellular anionic macromolecules are strongly bound to or form complexes with retroviruses and heat/protease resistant prion proteins which have been implicated in the spread of transmissible spongiform encephalopathies such as Creutzfeld-Jakob disease, kuru, Gerstmann-Straussler-Scheiner syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathies in cattle. (Weissmann C *et al*, Transmission of prions, www.pnas.org/cgi/doi/1073/pnas.172403799; Narang H, A critical review of the nature of the spongiform encephalopathy agent: protein theory versus virus theory, *Exp Biol. Med*, 2002, 227: 4-19; C, *et al*, The prion protein has DNA strand transfer properties similar to retroviral nucleocapsid protein, *J. Mol. Biol.*, 2001, 307: 1011-1021; Nandi PK and Sizaret P-Y, Murine recombinant prion protein induces ordered aggregation of nucleic acids to condensed globular structures, *Arch Virol*, 2001, 146: 327-45; Cominichini S, *et al*, Genomic organisation, comparative analysis and genetic polymorphisms of the bovine and ovine prion Doppel genes (PRND), *Mamm Genome* 2001, 9: 729-33,). These intracellular entities could therefore be consumed by the subject in appreciable amounts when they comply with the manufactures recommended dosage of one or more grams of ChS daily for the suppression of the symptoms arising from osteoarthritis and related conditions .

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the

field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

DISCLOSURE OF INVENTION

5 In a first aspect the invention provides a method for the preparation of a connective tissue-derived GAG-peptide complex and polypeptide said method comprising the steps of:

subjecting particles of connective tissue to enzyme mediated autolysis by contacting with an autolysis medium containing a monovalent or divalent salt at an effective pH and temperature such that at least one GAG-peptide complex and at least one polypeptide are released substantially free of DNA, by the proteolytic actions of an endogenous enzyme(s) from within the tissue particles into the autolysis medium, leaving residual tissue particles; and recovering the GAG-peptide complex(es) and polypeptide(s) from the autolysis medium.

15 Connective tissues are rich in collagens, non-collagenous proteins and proteoglycans. Connective tissue proteoglycans are macromolecular complexes consisting of chains made of sulfated polysaccharides (about 95%) covalently linked to a protein core (about 5%).

Glycosaminoglycan (GAG) refers to the polysaccharide chains of proteoglycans, which are composed of repeating disaccharide units containing a derivative of an amino sugar (either glucosamine or galactosamine) glycosidically linked to glucuronic or iduronic acid. The most common derivatives being o-sulfated esters substituted in the 4 or 6 positions of the N-acetylated glucosamine or galactosamine rings.

Examples of GAGs include hyaluronic acid (hyaluronan) (which is non-sulfated), chondroitin sulfate, keratan sulfate and heparan sulfate.

Cleavage of a proteoglycan structure produces complexes comprising one or more polysaccharide chains attached to a polypeptide fragment derived from the proteoglycan protein core. These complexes are referred to herein as "GAG-peptide complexes".

30 The term "polypeptide" includes peptides comprising two or more amino acids, but typically having 10 or more amino acids. Polypeptides can typically be derived by chemical or enzymatic cleavage of proteins, wherein proteins include glycoproteins and non-glycosylated proteins. The term polypeptides includes matrix derived polypeptides which may be obtained from cleavage of non-glycosylated or glycosylated proteins.

35 The properties of a polypeptide are determined by the type and sequence of its constituent amino acids.

The preferred monovalent salt is selected from hydrogen, sodium, potassium, ammonium and mixtures thereof, while the divalent salt is preferably selected from calcium, magnesium, copper, zinc and mixtures thereof. The more preferred salts are calcium and magnesium.

5 The pH may be in the range of from 2.5 to 8.5, preferably 3.5 to 8.0, more preferably 4 to 7 and most preferably 4.5 to 7.

The temperature may be in the range of from 20 to 45°C, preferably 25 to 45°C, more preferably 32 to 45°C, most preferably 37°C.

10 The invention also provides GAG-peptide complexes and polypeptides substantially free of DNA prepared by the method described above.

The invention still further provides monovalent or divalent salts of GAG-peptide complexes substantially free of DNA and polypeptides substantially free of DNA.

15 The connective tissue suitable for the method of the present invention includes skin, bone, tendon, ligament and cartilage. The source of connective tissue may be from a wide variety of species including bovine, ovine, porcine, equine, avian, cervine and piscine species. Cartilage is preferred and may be hyaline, elastic or fibro type cartilage. Preferred animal species and anatomical locations of cartilage are bovine, ovine, porcine, cervine or equine, tracheal, articular, auricular or nasal cartilage, chicken sternal or rib cartilage, shark skeletal cartilage and growing deer antler
20 cartilage. Tissues from young animals are preferable to those obtained from more mature, which in the case of the bovine species is greater than 3 years, for example.

25 The connective tissue may be treated and washed as required by methods known in the art to remove any adhering soft tissues and reduced to the required particle size by means including, but not limited to, mincing, dicing, grinding and the like. Appropriate particle size may be selected on the basis of commercial convenience. Preferred particle sizes are in the order of up to 5mm, preferably 1-3mm.

30 A person skilled in the art will appreciate that the rate of autolysis may vary with many factors including pH, temperature, concentration, tissue type, tissue particle size and time of incubation. In a preferred embodiment, cartilage particles of size 1-3mm are subject to autolysis in an aqueous medium at a pH of 4-5 and temperature of 32-42°C for up to 36 hours, preferably 16-24 hours.

35 The person skilled in the art will also appreciate that there are a number of methods for assaying for DNA content and that sample to sample variations, independent of method used, can be expected. The present inventors have used a DNA fluorescence dye binding assay and a direct spectroscopic method to estimate DNA levels in these preparations. The spectroscopic method is that normally utilized by

investigators to quantitate the levels of DNA in water soluble preparations which have a low protein content, while the fluorescence dye binding assay is employed to determine the amounts of DNA in samples which may contain proteins and other tissue derived molecules (Davis LG, Kuehl WM, Battey JF, Basic methods in Molecular
5 Biology, 2nd Edition, Appleton and Lange, Norfolk, Connecticut, 1994).

The term "substantially free of DNA" is to be understood in the context of the present invention to indicate a substantial reduction of DNA relative to the known DNA content of raw material (tissue particles prior to autolysis) or to commercial chondroitin sulfate preparations manufactured by alternative methods to those
10 described here. For example, the ratio of sulfated glycosaminoglycans (S-GAG) to DNA in three commercially available chondroitin sulfate preparations was found by the present inventors to average about 300:1 (Table 3). By contrast, GAG-peptides and polypeptides released into an autolysis medium in accordance with the present invention provide ratios of S-GAG to DNA in the autolysis medium of greater than
15 1000 to 1 and the DNA being undetectable by the DNA Hoechst 33258 fluorescence dye binding assay or spectroscopically. A person skilled in the art would appreciate that ratios of greater than 1000:1 would fall within the scope of the term "substantially free of DNA". By contrast, the ratio of S-GAG to DNA in commercially available ChSA for human consumption with ratios around 300:1 (Table 3) are considered by
20 the inventors not to be substantially free of DNA.

In the context of the present invention, DNA content is used as a marker for intra-cellular macromolecular components. For example, the substantial absence of DNA indicates a substantial absence of intra-cellular components in general such as nucleic acids and any associated molecules.

25 Methods of recovering the GAG-peptide complexes and polypeptides from the autolysis media are well known in the art. For example, the residual tissue particles are removed by filtration from the autolysis media and the GAG-peptide complexes and polypeptides isolated from the media. Suitable isolation methods include neutralisation of the supernatant followed by:

30 freeze drying; or
precipitation with acetone or aliphatic alcohols; or
by the formation of water insoluble complexes with quaternary ammonium salts such as cetyl, pyridinium, chloride; or
by separation using size exclusion or ion-exchange or other forms of column
35 chromatography or membrane filtration technology.

GAG-peptide complexes and polypeptides may be further purified and/or separated using conventional procedures. ChSs as their univalent (eg H, Na, K, ammonium) or divalent (eg Ca, Mg, Zn, Cu) salts obtained by mild chemical or enzymatic hydrolysis of the GAG-peptide complexes prepared by the methods
5 described herein are also substantially free of DNA, the ratios of S-GAG to DNA being greater than 1000: 1. Collagen peptides may be isolated by methods known in the art (US Patent 4,804,745 Feb 1989, US Patent 5,399,347 Mar 1995, US Patent 5,364,845 Dec 1996, US Patent 6,025,327 Feb 2000).

In a second aspect the present invention provides a Chondroitin sulfate salt
10 substantially free of DNA, derived from a GAG peptide complex , the GAG peptide complex being obtainable by the method described above.

The present invention also provides a monovalent or divalent salt of chondroitin sulfate substantially free of DNA wherein the monovalent salt is selected from the group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof,
15 and the divalent salt is selected from the group consisting of calcium, magnesium, copper, zinc and mixtures thereof.

GAG-peptide complexes substantially free of DNA, chondroitin sulfate salts substantially free of DNA and mixtures of GAG-peptide complexes with polypeptides substantially free of DNA may be used for the treatment, protection and restoration of
20 connective tissues in inflammatory and degenerative tissue disorders and as anti-angiogenic agents for the treatment of cancers or antilipideamic/profibrinolytic agents for improving blood flow in dysfunctional blood vessels.

In this regard the inventors were surprised to discover that the pharmacological activities exhibited by the GAG-peptide complexes substantially free of DNA and
25 GAG-peptide complex/polypeptide mixtures substantially free of DNA were superior to the corresponding activities of the commercially available ChS when their pharmacological activities were compared on an equivalent weight basis.

Accordingly, in a third aspect, the present invention provides use of: at least one GAG-peptide complex substantially free of DNA; and/or chondroitin sulfate salt
30 substantially free of DNA; at least one GAG-peptide complex substantially free of DNA and at least one polypeptide substantially free of DNA, for the manufacture of a medicament for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and
35 diabetic arteriopathies, thrombosis and embolisms, hyperlipideamis and for the treatment of cancers.

The present invention also provides a method for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers comprising the administration to a subject in need of such treatment an effective amount of at least one GAG-peptide complex substantially free of DNA; and/or chondroitin sulfate salt substantially free of DNA; at least one GAG-peptide complex substantially free of DNA and at least one polypeptide substantially free of DNA.

10 The chondroitin sulfates may be present as univalent (preferably Na, K, ammonium) or divalent (preferably Ca, Mg, Zn, Cu, more preferably Ca, Mg) salts.

In a fourth aspect the present invention provides a pharmaceutical composition comprising at least one GAG-peptide complex substantially free of DNA; and/or chondroitin sulfate salt substantially free of DNA; at least one GAG-peptide complex substantially free of DNA and at least one polypeptide substantially free of DNA, together with a pharmaceutically acceptable carrier. The chondroitin sulfates may be present as univalent (preferably Na, K, ammonium) or divalent (preferably Ca, Mg, Zn, Cu, more preferably Ca, Mg salts). The pharmaceutical compositions may include excipients known to be used in combination with chondroitin sulfate such as glucosamine.

In a preferred aspect the present invention provides use of a calcium salt of at least one GAG-peptide complex substantially free of DNA and/or a calcium salt of chondroitin sulfate substantially free of DNA for the manufacture of a medicament for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers.

The present invention also provides a method of treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers comprising the administration to a subject in need of such treatment an effective amount of a calcium salt of at least one GAG-peptide complex substantially free of DNA and/or a calcium salt of chondroitin sulfate substantially free of DNA.

The inventors have found that the use of a mixture of a calcium salt of a GAG-peptide complex and a calcium salt of a polypeptide generated by the method described above enhances connective tissue cell anabolism and cell biosynthesis of macromolecular components such as PGs and HA, even when the cells are exposed to suppressive proinflammatory cytokines and also together exhibit immunosuppressive and anti-inflammatory activities when administered to an animal model of arthritis.

Accordingly, in a fifth aspect the present invention provides use of a calcium salt of at least one GAG-peptide complex and a calcium salt of at least one polypeptide substantially free of DNA for the manufacture of a medicament for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers.

The present invention also provides a method of treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers comprising the administration to a subject in need of such treatment a calcium salt of at least one GAG-peptide complex and a calcium salt of at least one polypeptide substantially free of DNA. Preferably the calcium salt of the GAG-peptide complex is substantially free of DNA. The calcium salts of the GAG-peptide complexes and polypeptides may be co-isolated from connective tissue, preferably tracheal cartilage, more preferably bovine tracheal cartilage by the method described above.

In a sixth aspect, the present invention provides a pharmaceutical composition comprising a calcium salt of at least one GAG-peptide complex and a calcium salt of at least one polypeptide substantially free of DNA together with a pharmaceutically acceptable carrier. Preferably the calcium salt of the GAG-peptide complex is substantially free of DNA.

In a seventh aspect, the present invention provides use of at least one polypeptide substantially free of DNA for the manufacture of a medicament for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms.

The present invention also provides a method of treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of

their multiple forms comprising the administration to a subject in need of such treatment an effective amount of at least one polypeptide substantially free of DNA.

In an eighth aspect the present invention provides a pharmaceutical composition comprising at least one polypeptide substantially free of DNA together with a
5 pharmaceutically acceptable carrier.

The method of the first aspect of the present invention is essentially nondisruptive to the connective tissue used. Thus the residual tissue particles removed from the autolysis medium after release of the GAG-peptide complexes and polypeptides into the medium can be used as a source of collagen derivatives. In this
10 regard it should be noted that traditional methods for preparing ChS from connective tissues such as cartilage use exhaustive proteolytic or chemical digestion to destroy the proteinaceous components of the matrix to allow the ChS to be released into aqueous solution and isolated. The residual tissue particles obtained by the present method can be hydrolysed proteolytically or chemically and subsequently purified to obtain
15 collagen peptides of variable molecular size to be used for the treatment, protection and restoration of connective tissues in inflammatory and degenerative disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms or for the enhancement of wound healing or for the preparation of artificial biomatrixes for cell culture, cell transplantation, or delivery of bioactive compounds including drugs and
20 growth factors into a host tissue.

In a ninth aspect, the present invention provides a method according to the first aspect of the invention further including the step of hydrolysing the residual tissue particles to obtain collagen peptides.

The invention also provides collagen peptides prepared by the method described
25 above.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of
30 elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

BRIEF DESCRIPTION OF DRAWINGS

FIGURE 1

Kinetics of Glycosaminoglycan-peptide (GAG-peptide) Released from 1 mm and 3 mm
5 Bovine Trachea Cartilage Powders with 100mM with autolysis buffer, 100mM calcium
acetate, pH 4.5 at 37 degrees C.

FIGURE 2

Photomicrographs of histological sections of 3mm bovine tracheal cartilage powders
10 particles before and after subjecting them to the autolysis procedure using 100mM
calcium acetate buffer, pH 4,5 at 37 degrees C. Panels A and C are sections of
cartilage before autolytic processing and B and D after. Panels A and B show the
results of staining with Toluidine Blue (TB), a dye which binds to glycosaminoglycans
(GAGs) while C and D show sections stained with Masson Trichrome (Masson TC), a
15 dye known to stain native collagen fibres magenta colour. Note the loss of staining for
GAGs in section B after autolysis but a slightly increased intensity of staining for
collagen following the removal of the majority of GAGs as shown in D. Cell nuclei,
identified in these sections by the green counter-stains are clearly unchanged by the
autolytic process. All sections are shown at magnification X 400.

20

FIGURE 3

Kinetics of release of glycosaminoglycans-peptides (GAGs) from bovine tracheal
cartilage in the presence of 100mM calcium acetate at 37 degrees C at various pHs.

25 FIGURE 4

Kinetics of release of glycosaminoglycans-peptides (GAGs) from bovine tracheal
cartilage in the presence of 100mM calcium acetate at pH 4.5 C at various
temperatures.

30 FIGURE 5

Release of glycosaminoglycans-peptides (GAGs) from bovine tracheal cartilage in
100mM calcium acetate buffer at pH 4,5 C at 37 degrees C in the absence and presence
of proteolytic inhibitors. The cysteine protease inhibitor, N-Ethylmaleimide was the
most effective inhibitor reducing release by approximately 50% that of the control
35 incubations which contained no inhibitors.

FIGURE 6

Superdex-200 Gel Filtration of Calcium Peptacan (CaP) showing sulfated GAG (A_{535}) and protein (A_{562}) profiles using assays described in the text.

5 FIGURE 7

Superdex-200 Gel Filtration profiles of Calcium Peptacan (CaP) and Papain Digested CaP showing decrease in molecular size to that of chondroitin sulfate.

FIGURE 8

- 10 Superdex-200 Gel Filtration chromatograms of Peptacans (CaP and H2OP) and ChSAs (Sigma and Bioiberica).

FIGURE 9

- Standard Curve of distribution coefficient (K_{av}) versus molecular mass of ChS standards determined on Sephadex-G 200 using the method of Melrose and Ghosh (1993) as described in the text.
- 15

FIGURE 10

- Composite Agarose Polyacrylamide Gel (CAPAGE) Analysis of chondroitin sulfate (ChS) and Calcium Peptacans (CaP). Lane 1: ChS (Sigma), lane 2: CaP, lane 3: purified CaP, lane 4: papain digested CaP.
- 20

FIGURE 11

- Analysis of proteins and peptides in Peptacans Using SDS-Polyacrylamide gel electrophoresis. Lane 1= protein markers with molecular weights shown, lane 2 = CaP, lane 3 = purified CaP, lane 4 = chondroitinase-ABC digested and purified CaP.
- 25

FIGURE 12

- Determination of DNA contamination in ChSA and Peptacans from their UV Spectra. The presence of DNA is indicated by absorption at A_{260} and protein at A_{280} .
- 30

FIGURE 13

- Binding and release of Chondroitin Sulfate A (ChSA) and Calcium Peptacan (CaP) from immobilized lysozyme, hyaluronadase, and human granulocyte elastase using the BIAcore2000 technique. The extent of binding affinity of drugs to immobilized enzymes was recorded as resonance units (RU).
- 35

FIGURE 14

Short-Term (2 days) Concentration-Dependent Effects of Chondroitin Sulfate A (ChSA) and Calcium Peptacan (CaP) on the Incorporation of ^3H -methyl Thymidine into DNA of Ovine Chondrocytes in Monolayer Cultures.

FIGURE 15

Long-Term (7 days) Concentration-Dependent Effects of Chondroitin Sulfate (ChSA) and Calcium Peptacan (CaP) on the Incorporation of ^3H -methyl Thymidine into DNA of Ovine Articular Chondrocytes in Monolayer Cultures.

FIGURE 16

Concentration-Dependent Effects of Chondroitin Sulfate A (ChSA) and Calcium Peptacan (CaP) on Proteoglycan (PG) Synthesis by Ovine Chondrocytes.

FIGURE 17

Effects of Chondroitin Sulfate A (ChSA) and Calcium Peptacan (CaP) on Proteoglycan (PG) Synthesis by Ovine Chondrocytes in the Absence and Presence of Interleukin-1beta (IL-1beta).

FIGURE 18

Concentration Effects of ChSA and Peptacans on ^{35}S Incorporation into PGs of the Extracellular Matrix of Fibrochondrocytes isolated from the Inner Region of Ovine Menisci.

FIGURE 19

Concentration Effects of ChSA and Peptacans on ^{35}S -PGs released into condition medium from the Extracellular Matrix of Fibrochondrocytes isolated from the inner Region of Ovine Menisci.

FIGURE 20

Relative Effects of ChSA and Peptacans (2.0 mg/ml) on ^{35}S -incorporation into PGs of the Extracellular Matrix of Fibrochondrocytes of isolated from the inner Region of Ovine Menisci.

FIGURE 21

Relative Effects of ChSA and Peptacans (2.0 mg/ml) on ^{35}S -PGs released into conditioned medium from the Extracellular Matrix of Fibrochondrocytes isolated from the inner Region of Ovine Menisci.

FIGURE 22

Concentration Effects of ChSA and Peptacans on ^{35}S -incorporation into PGs of the extracellular matrix of Fibrochondrocytes isolated from the outer Region of Ovine Menisci.

FIGURE 23

Concentration Effects of ChSA and Peptacans on ^{35}S -PGs Released into Conditioned Media from Fibrochondrocytes isolated from the Outer Region of Ovine Menisci.

FIGURE 24

Relative Effects of ChSA and Peptacans (2.0 mg/ml) on ^{35}S -incorporation into PGs of the extracellular matrix of Fibrochondrocytes isolated from the outer Region of Ovine Menisci.

FIGURE 25

Relative Effects of ChSA and Peptacans (2.0 mg/ml) on ^{35}S -PGs Released into Conditioned Media from Fibrochondrocytes isolated from the outer Region of Ovine Menisci.

FIGURE 26

Concentration Effects of Chondroitin Sulfate A (ChSA) and Calcium Peptacan (CaP) on DNA Synthesis by cultures of Human Synovial Fibroblasts (HSF) from Joints of Osteoarthritic Patients. Concentrations of CaP between 62 and 250 micrograms/mL produced significant stimulation of synthesis ($p < 0.05$).

FIGURE 27

Effects of ChSA, CaP and H2OP (2.0 mg/ml) on HA Synthesis by Human Synovial Fibroblasts (HSF) as shown by the radioactivity profiles obtained on Superose 6 Gel Filtration chromatography.

FIGURE 28

Relative Effects of Chondroitin Sulfate A (ChSA) and Peptacans (2.0 mg/ml) on Hyaluronan (HA) Synthesis by Human Synovial Fibroblasts (HSF) Derived from Joints
5 of Osteoarthritic Patients.

FIGURE 29

Relative effects of Chondroitin Sulfate A (ChSA) and Peptacans and highly purified Peptacans (2.0 mg/ml) on Hyaluronan (HA) Synthesis by Human Synovial Fibroblasts
10 (HSF) Derived from Joints of Osteoarthritic Patients. Note the diminished activity of pCaP relative to CaP.

FIGURE 30

Superose-6 Gel Filtration chromatograms of media removed from cultures of synovial
15 fibroblasts obtained from joints of arthritic rabbits of non-drug treated controls (broken lines) and 4 animals treated daily with 300mg/kg of CaP for 14 days(solid lines) showing increased synthesis of radioactively labeled HA.

FIGURE 31

20 Mean (\pm SEM) group effects of ChS and CaP treatments on the ex-vivo synthesis of radioactively labelled HA by synovial fibroblasts from rabbit joints. Only cells from the CaP treated groups at 300mg/kg showed a significant increase in synthesis relative to the non-drug treated control group ($p < 0.05$).

25 FIGURE 32

Photomicrograph of histological sections of patella cartilage (AC) and adjacent synovium from joints of the rabbit PC arthritis model showing the extensive synovial inflammation and pannus formation accompanied by antibody staining for inducible nitric oxide synthase (iNOS) in chondrocytes. Panels A and B: sections from saline
30 injected joints but processed and stained with the iNOS primary and secondary antibodies as described in the text. Note the strong non-specific brown staining of synovial tissue but not chondrocytes (which appear blue from the counter-stain) or the cartilage matrix in these control sections. A=100X, B=400X magnification. Panels C and D are sections from PC injected non-drug treated joints. C=100X, D=400X
35 magnification. Intense staining for iNOS in chondrocytes is evident in these sections. Panel E is a section from PC injected joint of a ChS treated animal magnification X400.

Staining for expression of iNOS by chondrocytes is less than in the non-drug treated control but is still apparent. Panel F is a representative section from a PC injected joint of a CaP treated animal (300mg/kg) where staining for iNOS in chondrocytes is reduced relative to control and ChS treated groups. Magnification X400.

5

FIGURE 33

- Photomicrograph of histological sections of patella cartilage and adjacent synovium from joints of the rabbit PC arthritis model showing the extensive synovial inflammation and pannus formation accompanied by brown antibody staining in chondrocytes for the nitric oxide synthase oxydation product, nitrotyrosine. The section shown in Panel A is from PC injected non-drug treated rabbit joints process and stained with the primary and secondary bodies for nitrotyrosine as described in the text. Note as in FIGURE 32 the strong brown non-specific staining of synovial tissue but specific staining for the antigen in chondrocytes, particularly in the cartilage superficial zone.
- 15 Magnification X400. Panel B shows a representative section from a PC injected joint of a ChS treated animal where staining is reduced in intensity but not absent. Magnification X400. Panel C is a section from a PC injected joint of an animal given CaP (300mg/kg). Magnification X200. Staining for nitrotyrosine in chondrocytes and in the superficial cartilage zone was slight and less than in the non-drug treated control.
- 20 Panel C is a negative control section from a PC injected joint which confirms the non specific binding of the nitrotyrosine antibody to synovial tissues but not cartilage or chondrocytes.

FIGURE 34

- 25 Histograms showing the mean values (\pm SEM) for neutrophils in the blood of animals from the ChS and CaP treated rabbit groups. Note the suppression of neutrophil levels in blood from the high dose CaP treated group.

FIGURE 35

- 30 Histograms showing the mean values (\pm SEM) for mononuclear cells in the blood of animals from the ChS and CaP treated rabbit groups. Note the reduction of mononuclear cells levels in the 300 and 200mg/kg CaP treated groups.

MODES FOR CARRYING OUT THE INVENTION

Abbreviations and definitions:

In order to avoid repetition and improve the clarity of this description of the products isolated from cartilages by the methods of the invention they have been
5 abbreviated and given the collective title of PEPTACANS (P). The different Peptacans isolated by the methods herein are identified by the addition of prefixes, Thus:

Calcium Peptacan (CaP) - is the aqueous soluble product obtained by subjecting bovine tracheal cartilage powder to autolysis at 37°C with 0.1 M calcium acetate at pH
10 4.5.

Sodium Peptacan (NaP) - is the aqueous soluble product obtained by subjecting bovine tracheal cartilage powder to autolysis at 37°C with 0.1 M sodium acetate at pH
4.5.

Water Peptacan (H2OP) - is the aqueous soluble product obtained by subjecting
15 bovine tracheal cartilage powder to autolysis at 37°C with dilute acetic acid pH at 4.5.

dCaP - is CaP dialysed against H₂O

dNaP - is NaP dialysed against H₂O

dH2OP - is H₂OP dialysed against H₂O

pCaP - is CaP purified by gel filtration or ion exchange chromatography

20 pNaP - is NaP purified by gel filtration or ion exchange chromatography

pH2OP - is H2OP purified by gel filtration or ion exchange chromatography

Experimental Protocols

25 Bovine, ovine, cervine or porcine tracheal cartilage or nasal cartilage, chicken sternal cartilage, or skeletal shark cartilage or deer antler cartilage were freed of adhering soft tissues mechanically or as described previously US Patent 5,399,347 Mar 1995, US Patent 5,364,845 Dec 1996, US Patent 6,025,327 Feb 2000). These cleaned hyaline cartilages were rinsed with water, minced into 1 mm or 3 mm sizes, freeze
30 dried and stored at -20 °C. Bovine tracheal chondroitin sulfate A (ChSA) was purchased from Sigma Chemical Co, USA or was obtained as a gift from Bioiberica, Barcelona, Spain (batch 1/0015, batch 05/2001, batch 18/11/99). All other chemicals were of analytical grade and were purchased from local suppliers.

Release of Glycosaminoglycan Peptide (GAG-peptide) Complexes and polypeptides from the Cartilage Powders

Studies on the kinetics of release of the GAG-peptides and polypeptides from the cartilage powders using the different buffers (eg sodium or calcium acetate or dilute acetic acid to give the various Peptacan products listed above in abbreviations was undertaken under a variety of conditions. The objective of these experiments was to determine the effects of (i) particle size - 3mm, 5mm, (ii) different pHs eg. pH range 3.5-7.0, (iii) different temperatures, 4°C, 25°C and 37°C, and (iv) animal species and tissue locations on the rate of autolysis and product release into the aqueous phase. All the experiments were performed, with stirring and release of sulphated GAGs and polypeptides monitored over 24 hours. Studies on the kinetics of release of the GAG-peptide complexes from the cartilages showed that more than 80% of the GAG content could be mobilised into the aqueous medium after 24 hours. Studies also showed that the rate of release was dependent on the cartilage particle size, the smaller preparations undergoing more rapid release. However, by 24 hours the yields obtained were the same. The pH and temperature were found to be important determinants of the rate of release which indicated that the release process was mediated by endogenous enzymes present within the solid tissues. This proposed mechanism was confirmed by undertaking autolysis experiments in the absence and presence of specific enzyme inhibitors. Since it was found that the addition of N-ethylmaleimide produced the most significant inhibition of GAG-peptide and polypeptides release into the aqueous medium we consider that the cysteine class of proteinases, such as the Cathepsins, were the major, but not exclusive, contributors to the autolytic process.

In all experiments the aqueous phase was separated from the cartilage powders by filtration and the filtrate centrifuged to remove fine particles and then neutralized to pH 7.0 by addition of an alkaline solution containing the desired cation. These Peptacan solutions after chemical analysis were either freeze dried and used directly for pharmacological studies or purified and/or converted to chondroitin sulfates. The freeze dried Peptacans were also used as stock material for the preparation of dialysed and fractionated preparations as described below. Alternatively the Peptacans could be isolated from the aqueous solutions obtained from the cartilage digests by precipitation with excess quantities of acetone, ethanol or methanol, usually by adding 3-5X the volume of the aqueous extracts. The precipitates so obtained would be washed with absolute ethanol and dried under vacuum then stored in a vacuum dessicator.

Histological examination of bovine tracheal cartilage samples before and after calcium acetate autolysis.

Minced 3 mm cartilage samples before and after treatment with 0.1M calcium acetate, pH 4.5 at 37°C for 24 hours were fixed in 10% (v/v) neutral buffered formalin for 48 hours. Specimens were then washed, dehydrated in increasing alcohol concentrations (70 – 100% v/v), and double-embedded in methyl benzoate / celloidin then paraffin wax. Sections (4µm) were cut on a rotary microtome and adhered to Superfrost Plus (Menzel Glaser, USA) glass slides. Histochemical staining with Toluidine blue (TB), a dye which binds to sulfated GAGs and Masson trichrom (TC), a dye that binds to native collagen, were performed in batches under controlled conditions as described previously (Hwa S-Y, Burkhardt D, Little C, Ghosh P, The effects of Diacerein on cartilage and subchondral bone in an ovine model of osteoarthritis, J Rheumatology, 2001, 28: 825-834). Briefly, sections were de-paraffinised, and equilibrated in 70% (v/v) alcohol for 15 minutes, then stained in 0.04% (w/v) Dye / 0.1M sodium acetate buffer (pH 4.0) for 10 minutes. They were then counter-stained in 0.1% (w/v) fast green for 2 minutes, dehydrated in isopropanol followed by xylene and cover-slips applied.

Purification of Glycosaminoglycan Peptide Samples by Gel Filtration

The freeze-dried Peptacans were dissolved in H₂O to afford concentrations of 4.0 mg/ml of clear solutions. 1.0 ml of above Peptacan solutions was injected into a pre-equilibrated HiLoad 16/60 Superdex 200 column and eluted with 0.5 M NaCl at the flow rate of 1.0 ml/min. Fractions (2.0 ml) were collected in 5-ml plastic tubes. GAG-peptide and protein content of each fraction was determined using sulphated glycosaminoglycan (S-GAG) (Farndale RW, Buttle DJ and Barrett AJ. Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. Biochim. Biophys. Acta: 883, 173-177) and BCA assay (Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC. Anal. Biochem. 150, 76 – 85, 1985) respectively. The fractions which were S-GAG-positive were pooled and freeze dried. These were designated as pCaP or pNaP or pH2OP.

Sulfated Glycosaminoglycan (S-GAG) DMMB Assay

The total S-GAG content of Peptacans was determined by reaction using the metachromatic dye 1,9-dimethylmethylene blue (DMMB) (Farndale RW, Buttle DJ and Barrett AJ. Improved quantitation and discrimination of sulfated glycosaminoglycans

by use of dimethylmethylene blue. Biochim. Biophys. Acta: 883, 173-177). A standard curve was prepared using a commercially available chondroitin sulfate A (ChSA) derived from bovine tracheal cartilage (ICN, USA) in 96-well microtitre plates. ChSA standard and Peptacan samples were diluted in 0.2% sodium formate before DMMB reagent was added and the absorbance at 535 nm read immediately. Softmax software was used to construct a standard curve and calculate the concentration of S-GAG in Peptacans.

Analysis of CaP Using High Performance Liquid Chromatography (HPLC)

- 10 The ratio of chondroitin-4-sulfate (Ch4S) and chondroitin-6-sulfate (Ch6S) isomers in Peptacans were determined using high performance liquid chromatography (HPLC) basically as described by Lee and Tieckelman (Lee GJL and Tieckelman H. The application of high performance liquid chromatography in enzymatic assays of chondroitin sulphate isomers in normal urine. J Chromatography. 222: 23-31, 1981).
- 15 Freeze dried CaP was dissolved in H₂O and then digested by incubation with 0.125 units of chondroitinase ABC (SKK, Tokyo Japan) at 37°C overnight. The unsaturated disaccharides were separated in a high performance amino column using 0.2 M ammonium acetate, pH 5.5 as mobile phase and photometrically detected at 232 nm. Peaks were identified by comparison with those of chondroitin sulphate A (Sigma
- 20 Chemical Co, USA). The area of each peak was measured using NIH Image 1.61.1 software.

Determination of collagen or collagen peptide content in preparations by assay for hydroxyproline

- 25 The collagen content of cartilage powder, freeze dried Peptacans or cartilage residue after extraction was estimated by measuring the concentration of the amino acid hydroxyproline which is unique to this protein. Each freeze dried Peptacan sample was directly dissolved in H₂O (10 mg/ml) and then hydrolysed in 5 N HCl at 110 °C for 24 h. The unprocessed cartilage powders or residues were papain digested for 24 h first
- 30 and then centrifuged and the supernatant collected, which was then subjected to 5N HCl hydrolysis as described above. The hydrolysed sample solution was neutralised to pH 7 before dilution and analysis. The hydroxyproline concentration in these solutions was determined using the method of Stegman and Stalder (Stegman H and Stalder K. Determination of Hydroxyproline. Clin. Chim. Acta 18: 267-273, 1967) by using a L-
- 35 hydroxyproline standard and measuring the absorbance at 562 nm after the addition of chloramine T and p-dimethylaminobenzaldehyde to develop the chromophore. The

hydroxyproline concentration was multiplied by 7.4 to give an estimate of the collagen content.

Determination of Protein content of preparations by the Bicinchoninic Acid (BCA)

5 assay

The total protein content of cartilage powder, freeze dried Peptacans or cartilage residue after extraction was determined using BCA assay (Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC. Anal. Biochem. 150, 76 – 85, 1985). Cartilage powder and
10 cartilage residue were papain-digested for 16 h and centrifuged to provide clear supernatants. Each freeze dried Peptacan was directly dissolved in H₂O to provide a 2.0 mg/ml solution. 20 µl of each sample solution was added to a well of 96-well plates. Just prior to assay, 50 parts of reagent 1 (0.4% NaOH; 1.7% Na₂CO₃; 0.95% NaHCO₃; 1.0% bicinchoninic acid; 0.16% Na₂-tartrate) was mixed with reagent 2 (4%
15 CuSO₄·5H₂O). 200 µl of this working reagent was added to the sample solution. After incubation at 37 °C for 60 min the absorbance A₅₆₂ was read using a Thermomax microplate reader. Bovine serum albumin (BSA) or highly purified gelatine (Sigma Chemical Co) at 0 – 10 µg/well were used to construct a standard curve.

20 Composite Agarose Polyacrylamide Gel Electrophoresis (CAPAGE)

Each of ChSA and Peptacan samples (CaP, pCaP or papain digested CaP) were dissolved in H₂O at the concentrations of 1.0 – 3.0 mg/ml and then mixed 1:1 with CAPAGE sample loading buffer (20 mM Tris-acetate, pH6.3, 1 mM Na₂SO₄, 60% sucrose and 0.01% bromophenol blue). 20 µl of each sample equivalent to 10 µg of
25 GAG was loaded into wells of 2 mm thick CAPAGE gel (0.6% agarose, 1.2% acrylamide, 10 mM Tris-acetate pH 6.3 and 0.25 mM sodium sulfate) and electrophoresed in the CAPAGE running buffer (10 mM Tris-acetate pH 6.3, 0.25 mM Na₂SO₄) at 150 V for 2 h. The gel was stained in a solution of 0.02% toluidine blue in 0.1 M acetic acid for 1 h, destained in 0.5 M acetic acid for 2 h and dried on an agarose
30 gel-bound film. The dried gel was rinsed with H₂O and dried again.

Determination of Average Molecular Size of Peptacans Using Gel Filtration chromatography

A HiLoad Superdex-200 prep grade preppacked column (16 mm x 60 mm) and
35 previously characterised ChS standards were used to determine the molecular size of the various Peptacans and their digested products. The column was equilibrated with

0.25 M NaCl for at least 3 h prior to loading samples. Sephadex-G200 chromatographed bovine tracheal chondroitin sulfate fractions (CS1 – CS7) prepared previously were used as molecular size standards (Melrose J and Ghosh P, Determination of the average molecular size of glycosaminoglycans by fast protein liquid chromatography. J Chromatography, 1993, 637; 91-95). 1.0 ml of standard (0.5 mg/ml) or Peptacans (CaP, H₂OP and pCaP) (1.0 mg/ml) were loaded into columns and chromatographed at a flow rate of 1 ml/min using 0.25 M NaCl solution. Fractions (1.0 ml) were collected in 5-ml plastic tubes. GAG content of each fraction was determined with S-GAG assay. A standard curve was constructed using molecular mass versus distribution coefficient (K_{av}) of CS1-CS7.

Analysis of Proteins in Peptacans by SDS-PAGE Electrophoresis

ChSA (Sigma) and CaP were dissolved in H₂O and then mixed 1:1 with 2 x sample loading buffer (0.07 M TrisHCl, 1.5% SDS, 20% glycerol, 0.2M DTT and 0.1% BPB) to achieve the final concentrations of 4.0 – 20 mg/ml. The samples were boiled in a water bath for 5 min. 20 µl of above samples were loaded into the wells of 8 – 16 % pre-cast Tris-glycine gel (Norvex). SeeBlue pre-stained low molecular weight range protein markers (Norvex) were loaded into wells on the left-hand side of the gel and electrophoresis was performed at 125 V for 2 h. The gel was stained in Coomassie blue R250 solution (40% ethanol, 10% acetic acid and 0.2% Coomassie R250) for 30 min and destained in a solution containing 10% ethanol and 7.5% acetic acid for 16 h. The gel was dried in a Bio-Rad Gelair drier.

Determination of DNA in Peptacans and Commercial Chondroitin Sulfates Using UV Spectroscopy

Chondroitin sulfates (Bioiberica or Sigma) and Peptacans (CaP, NaP and H₂OP) were prepared at 1.0 mg/ml in H₂O. 100 µl of each sample solution were loaded into a micro-cuvette and scanned spectrophotometrically over the wavelength range of 220 – 320 nm. The absorbance curves, A₂₆₀ and A₂₈₀ were recorded for each sample in triplicate. The absorption of light by concentrated aqueous solutions of these preparations at the wavelength of 260nm in their UV spectra provides a measure of the levels of DNA present; whereas absorption by these solutions at 280nm is taken as a measure of protein content. The ratio of the A₂₆₀ and A₂₈₀ values is commonly used as an index of DNA purity, with ratios of more than 1.5 being considered to indicate preparations high in DNA.

Determination of DNA in Peptacans and Commercial Chondroitin Sulfates Using the Hoechst 33258 dye binding assay

Chondroitin sulfates (Bioiberica or Sigma) and Peptacans (CaP, NaP and H2OP) were prepared at 1.0 mg/ml in H₂O and the DNA content was determined in duplicate by using a fluorometric assay in which the Hoechst 33258 dye on binding to DNA shows a change in fluorescence (Kim YJ, Sah RLY, Doong J-YH, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem. 1988;174:168-176). For the cartilage preparations prior digestion with papain was employed as described by Kim *et al* (1988) in the in the above publication. Briefly, DNA was determine by adding 100 µl/well of Hoechst 33258 dye solution to each well of a 96-well plate followed by adding 100 µl of ChS or Peptacan or papain digested samples. The plate was gently agitated for 5 min before measuring the fluorescence using excitation and emission wavelengths of 350 nm and 450 nm respectively and slit widths of 10 nm and 15 nm. Calf thymus DNA (Sigma Chemical Co)(0-25 µg/ml) with a uv spectral ratio of A₂₆₀ to A₂₈₀ of 1.85 was used to construct a standard curve and the DNA content of the preparations determined relative to this standard. DNA results were expressed as a % of the dry weight of the samples.

Determination of Binding Affinity of CaP and ChSA to Lysozyme, Elastase and Hyaluronidase Using BIAcore 2000

The molecular interactions between CaP or ChSA and lysozyme, elastase or hyaluronidase were investigated using a surface plasmon resonance (SPR) biosensor device – BIAcore 2000 system (Pharmacia Biosensor AB). Lysozyme (chicken egg-white, CalBiochem), elastase (human neutrophil, ICN) or hyaluronidase (bovine testes type IV, Sigma) was dissolved in 100 µg/ml in 10 mM sodium acetate, pH 6.0 and immobilized on a CM5 sensor chip by using amine coupling procedure. CaP or ChSA was firstly dissolved in H₂O and then diluted in standard HBS running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) to 2.5 µg/ml. The binding of CaP or ChSA to immobilized enzymes was determined in four flow cells separately including one of the flow cells (minus ligand) used to record background sensorgrams. 200 µl of CaP or ChSA solution were injected individually on to the immobilized chip surface and the molecular interactions were monitored at the flow rate of 50 µg/ml at 25 °C. The sensorgrams were recorded and evaluated using the BIAevaluation 3.1 software provided with the system.

Inhibition of Human Neutrophil Elastase (HNE) by Peptacans and Chondroitin Sulfate

A

Stock solutions of chondroitin sulfate A (ChSA) and Peptacans were prepared at 2.5 mg/ml in HNE assay buffer (50 mM sodium phosphate, pH 7.4, 0.1% BSA, 0.025% Triton X100) and serially diluted to give solutions with concentrations within the range 4.9 – 1250 µg/ml. Triplicate diluted samples (100µl) were transferred to wells of a microtitre plate to which was added 50 µl of 2.0 µg/ml human neutrophil elastase (HNE) in the HNE assay buffer and the plate incubated at 37 °C for 10 min. Substrate solution (100µl of 0.5 mM SAAVNA in 12.5% DMSO and 87.5% assay buffer) were added to each well and the absorbance was read at 405 nm 4 times at 30 minute intervals. The elastase inhibition rates were expressed as a percentage of the control rate and a plot of % residual HNE activity versus inhibitor concentrations in the inhibition assay was prepared.

15 Ovine Chondrocyte Cultures

Freshly prepared or cryopreserved primary ovine articular chondrocytes were removed from liquid nitrogen storage and were immediately thawed at 37 °C in a water bath and rinsed twice in culture medium containing 50 µg/ml gentamicin. The cells were seeded at the density of 2×10^5 cells /well in 6-well plates and grown in an atmosphere of 5% CO₂ in air at 37 °C for 4 days. Medium was changed every 2 days.

Effects of Peptacans and Chondroitin Sulfate A on DNA Synthesis by Ovine Chondrocytes Using ³H-methyl Thymidine Incorporation Assay

DNA synthesis in ovine chondrocytes stimulated with chondroitin sulfate A (ChSA) or Peptacans (CaP, NaP or H2OP) was determined in duplicate by the incorporation of ³H-methyl thymidine into this nucleic acid. The cell cultures were maintained in monolayer culture in 2.0 ml of DMEM/F12 medium containing 10% FCS and 50 µg/ml gentamicin for 3 days before adding the samples. ChSA or Peptacans were added at the concentrations of 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µg/ml in the above medium and incubated in an atmosphere of 5% CO₂ in air at 37 °C for 24 h (short-term) or 6 days (long-term) followed by incubation with 0.25 µCi/well ³H-methyl thymidine for a further 24 h. Cells were then harvested and radioactivity in DNA was determined using a β-counter. Data were expressed as mean values ± SEM.

Effects of Peptacans and Chondroitin Sulfate A on DNA Synthesis Using a fluorometric Assay

DNA synthesis in human synovial fibroblasts (HSF) was determined in duplicate by using fluorometric assay with Hoechst 33258 dye. The HSF, from joints of
5 osteoarthritic patients, was cultured in monolayer in 6-well plates for 24 h. Then the serial concentrations of ChSA or Peptacans were added at 62.5, 125, 250, 500, 1000 and 2000 $\mu\text{g/ml}$ in DMEM medium containing 10% FCS and 50 $\mu\text{g/ml}$ gentamicin. The cultures were maintained in an atmosphere of 5% CO_2 in air at 37 °C for a further 24 h. The cells were detached by trypsin/EDTA (0.2%/0.1%) treatment and then papain
10 digested. 100 μl /well of Hoechst 33258 dye solution was added to a 96-well plate followed by adding 100 μl of papain digested samples. The plate was gently agitated for 5 min before measuring the fluorescence using excitation and emission wavelengths of 350 nm and 450 nm respectively and slit widths of 10 nm and 15 nm. Calf thymus DNA (0-25 $\mu\text{g/ml}$) was used as a standard.

15

Effects of Peptacans and Chondroitin Sulfate A (ChSA) on Proteoglycan (PG) Synthesis by Ovine Chondrocytes using a $^{35}\text{SO}_4$ – Incorporation Assay

The primary ovine articular chondrocytes prepared as described above were seeded at a density of 2×10^5 cells/well in 6-well plates and grown for 4 days before
20 addition of 2.0 ml of different concentrations ChSA or Peptacans (CaP, NaP or H_2OP) in DMEM/F12 culture medium containing 10% FCS and 50 $\mu\text{g/ml}$ gentamicin. Compounds were added in the presence or absence of 10 ng/ml recombinant human IL-1. The cultures were grown for further 3 days. Then 40 μCi /well $^{35}\text{SO}_4$ were added 24 h prior to the termination of cultures. After a further 24 h incubation at 37 °C,
25 conditioned media were collected in microfuge tubes. The cells were rinsed with cold PBS twice and then trypsinized with 0.2% trypsin/0.1% EDTA solution. The cell suspension was collected and centrifuged at 1310 x g for 5 min.

Papain digestion to release $^{35}\text{SO}_4$ labelled GAGs was performed by adding 0.5 ml of papain digestion buffer (2 $\mu\text{l/ml}$ papain suspension, 5 mM cysteine in PBS pH
30 6.2) into 0.5 ml of conditioned media, while the cell pellets and their extracellular matrix were directly resuspended in 0.5 ml of papain digestion buffer and incubated at 60 °C overnight. Newly synthesised ^{35}S Sulfated PGs (as ^{35}S -GAGs) were separated from free $^{35}\text{SO}_4$ by the following procedure: To 0.4 ml of each papain digested sample, 0.2 ml of 0.2 M Na_2SO_4 plus 50 mg/ml CSA (1:1) was added and vortexed. Then 0.1 ml of
35 0.4 M BaCl_2 was added to each tube and vortexed. The mixture was centrifuged at 1310 x g for 10 min. 0.4 ml of the supernatant was transferred into another tube. 0.1 ml

of 50 mg/ml CSA plus 0.4 M BaCl₂ (2:5) mix was added. Then 0.1 ml of 0.2 M Na₂SO₄ was added and centrifuged at 1310 x g for 10 min. The last step was repeated once. 0.1 ml of the supernatant was transferred into a pony vial followed by adding 3 ml / vial Eco-lite scintillation fluid and thoroughly mixed. Samples were then counted
5 in an automated β -counter (Canberra Packard 1500 Liquid Scintillation Analyzer).

Ovine Fibrochondrocyte Cultures

The skin and overlying musculature of ovine stifle joints were removed and the external joint capsule sprayed with 70% ethanol. The joints were then opened in a
10 lamina flow hood. The menisci were removed, rinsed with sterile PBS and cut into two parts – the inner and outer regions. The inner and outer meniscus tissues were individually dissected into small pieces and digested separately with 0.2% pronase in culture medium (DMEM containing 10% FCS and 50 μ g/ml gentamicin) for 2 h followed by 0.04% collagenase digestion overnight.

15 The digests were filtered 3 times through 70 μ m mesh and rinsed with DMEM culture medium containing 50 μ g/ml of gentamicin. The cells were seeded into 75 cm flasks and incubated in an atmosphere of 5% CO₂ in air at 37 °C for 7 days to reach confluency. The cells were rinsed twice with sterilised PBS, trypsinized with 0.2% trypsin/0.1% EDTA solution and rinsed twice with culture medium. The cells were
20 subcultured at the density of 2×10^5 cells /well in 6-well plates and grown for 2 days in an atmosphere of 5% CO₂ in air at 37 °C.

The Effects of Peptacans and Chondroitin Sulfate A on Proteoglycan (PG) Synthesis by Fibrochondrocytes using the ³⁵SO₄ – Incorporation Assay

25 The first passage fibrochondrocytes from either inner or outer region of ovine meniscus were seeded at the density of 2×10^5 cells /well in 6-well plates and grown for 2 days before 2.0 ml of different concentrations of chondroitin sulfate A (ChSA) or Peptacans (CaP, NaP or H2OP) in DMEM/F12 was added. The cultures were grown for a further 3 days then 40 μ Ci/well ³⁵SO₄ was added 24 h prior to the termination of
30 cultures. After 24 h incubation at 37°C, conditioned media were collected in microfuge tubes. The cells were rinsed with cold PBS twice and then trypsinized with 0.2% trypsin/0.1%EDTA solution. The cell suspension was collected and centrifuged at 1310 x g for 5 min.

Papain digestion was performed by adding 0.5 ml of papain digestion buffer (2
35 μ l/ml papain, 5 mM cysteine in PBS pH 6.2) into 0.5 ml of conditioned media, while the cell pellets were directly resuspended in 0.5 ml of papain digestion buffer and

incubated at 60 °C overnight. Newly synthesised ³⁵Sulfated PGs (as³⁵S-GAGs) were separated from free ³⁵SO₄ by the following procedure: To 0.4 ml of each papain digested sample, 0.2 ml of 0.2 M Na₂SO₄ plus 50 mg/ml CSA (1:1) was added and vortexed. Then 0.1 ml of 0.4 M BaCl₂ was added to each tube and vortexed. The mixture was centrifuged at 1310 x g for 10 min. 0.4 ml of the supernatant was transferred into another tube. 0.1 ml of 50 mg/ml CSA plus 0.4 M BaCl₂ (2:5) mix was added. Then 0.1 ml of 0.2 M Na₂SO₄ was added and centrifuged at 1310 x g for 10 min. The last step was repeated once. 0.1 ml of the supernatant was transferred into a pony vial followed by adding 3 ml/vial Eco-lite scintillation fluid and thoroughly mixed. Samples were then counted in an automated β-counter (Canberra Packard 1500 Liquid Scintillation Analyzer).

Human Synovial Fibroblast Cultures

Cryopreserved human synovial fibroblast (HSF) derived from joints of patients with osteoarthritis were thawed quickly at 37 °C in a water bath. The entire cell suspension in cryo-vials was transferred to a 15 ml sterile centrifuge tube using a transfer pipette in a lamina flow hood. The tubes were centrifuged at room temperature, at 800g for 10 min. The supernatant was discarded and the cell pellet completely resuspended in 10 ml DMEM medium containing 10% FCS and 50 µg/ml gentamicin by aspirating with a sterile transfer pipette. The tubes were re-centrifuged at 800g for 10 min at room temperature. The supernatant was again discarded and the cell pellet completely resuspended in 10 ml DMEM complete medium. The cell suspension was seeded in appropriate numbers of labelled flasks followed by the addition of 15 ml of DMEM complete medium. The cells were incubated in an atmosphere of 5% CO₂ in air at 37 °C until confluency. Culture medium was changed every 2 days. To subculture the cells, the flasks were removed from the 37°C incubator and the cells were rinsed twice at room temperature with PBS. Trypsin/EDTA (0.2/0.1%) solution was added and swirled over the cell layer. The flasks were recapped and maintained at 37 °C for 3 – 5 min. The cells were checked under a microscope for detachment. DMEM complete medium (2 – 3 ml per flask) was added and washed over the surface of flasks to collect the released cells. The cells were transferred to centrifuge tubes and centrifuged at 800g for 10 min at room temperature. The cells were washed twice and then resuspended in 10 ml DMEM complete medium. The cells were then counted using a haemocytometer. The cells were seeded at the density of 1.5 x 10⁵ cells /2 ml/well in 6-well plates and grown for 24 h in an atmosphere of 5% CO₂ in air at 37 °C.

Effects of Peptacans and Chondroitin Sulfate A on Hyaluronan(HA) Synthesis by Human Synovial Fibroblasts (HSF) Using a ^3H -glucosamine Incorporation Assay

The HSF cells were seeded at 1.5×10^5 cells/well in the 6-well plates and allowed to attach for 24 h before addition of compounds. Chondroitin sulfate A (ChSA) and Peptacans (CaP and H2OP) solutions were prepared in DMEM culture medium containing 10% FCS and 50 $\mu\text{g/ml}$ gentamicin at double the concentration required in the cultures. The highest concentration prepared was 4.0 mg/ml which was sterilised through a 0.22 μm filter and then serially diluted to give final concentrations of 2000, 1000, 500, 250, 125 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$ of compounds. One ml of each drug solution was added to each well of 6-well plate. Stock ^3H -glucosamine was diluted in culture medium to give a 1 $\mu\text{Ci/ml}$ solution which was immediately added to each well (1 $\mu\text{Ci/well}$). The plates were incubated for a further 24 h. At culture termination, media was collected into 5 ml capped tubes and stored at -20°C for ^3H -HA analysis.

Isolation and Quantitation of ^3H -Hyaluronan (^3H -HA) in Cultures Using Superose 6 Gel Filtration Chromatography

Two aliquots of 0.5 ml from each media sample were labelled A and B. 20 μl of 1 M acetic acid, pH 6.0 was added to all aliquots. 50 μl of reaction buffer (20 mM Na-acetate and 0.15 M NaCl, pH 6.0) was added to aliquot A and 50 μl of 5 TRU Streptomyces hyaluronidase in reaction buffer was added to aliquote B. All samples were incubated at 60°C for 3 h followed by boiling for 5 min to inactivate the added hyaluronidase. The samples were store at -20°C prior to gel filtration.

A gel filtration column prepacked with Superose 6 was used to isolate and identify ^3H -HA in culture media. Media samples were routinely centrifuged at high speed on a bench Microfuge for 10 min immediately before loading to the column. Samples (200 μl of each) were injected into the column through sample loop and the column was eluted with PBS buffer (0.15 M NaCl, 0.05 M Na_2PO_4 , pH 7.2) at the flow rate of 0.2 ml/1 min. The column eluent was collected at 0.5 ml/fraction for total of 46 fractions and radioactivity was determined using a β -scintillation counter.

Studies on the effects of oral administraion of Chondroitin Sulfate or Calcium Peptacan on joint inflammatory mediators, white blood cell numbers and *ex-vivo* synovial fibroblast biosynthesis of Hyaluronan in a rabbit model of arthritis.

The rationale, methodology and pathological outcomes obtained for the rabbit model of arthritis used in the present experiments has been described in detail elsewhere (Page-Thomas DP: Aspects of synovial degradation. Bayer-Symposium,

Experimental models of chronic inflammatory disease, 353-365 (1977), Springer-Verlag, Berlin; Cambray *et al.*, The effects of dexamethasone in vitro on the production of collagenase and inhibitor by synovial and cartilage explants from joints of rabbits with a proliferative arthritis, *Rheumatol Int*, 1981, 1: 69-72; Smith *et al.*, The effects of orally administered calcium pentosan polysulfate on inflammation and cartilage degradation produced in rabbit joints by intraarticular injection of a hyaluronate-polylysine complex. *Arthritis and Rheumatism*, 1994, 37: 125-136). In the present studies, male New Zealand White rabbits of approximately 4 months of age were used and acclimatised to holding cages for 7 days. Animals were then divided into 5 experimental groups according to the criteria provide in the following protocol.

Experimental Protocol:

Group A (n = 4) Non-drug-treated, Polycation(polylysine)-Hyaluronan complex (PC)-injected intra-articularly into one rabbit knee joint and an equal volume of isotonic saline injected in the contralateral knee joint on day 7.

Group B (n = 4) PC-injected intra-articularly into one knee joint and isotonic saline injected into the contralateral knee joint but animals administered 300mg/kg (body weight) chondroitin sulfate (Bioiberica batch # 18/11/99) orally daily for 14 days beginning 7 days before the PC injection.

Group C (n = 4) PC-injected intra-articularly in one knee joint and isotonic saline injected in the contralateral knee joint, administered 300mg/kg (body weight) CaP orally daily for 14 days beginning 7 days before PC injection.

Group D (n = 4) PC-injected intra-articularly in one knee joint and isotonic saline injected in the contralateral knee joint but animals administered 200mg/kg (body weight) CaP orally daily for 14 days beginning 7 days before PC injection.

Group E (n = 4) PC-injected intra-articularly in one knee joint and isotonic saline injected in the contralateral knee joint but animals administered with 100mg /kg (body weight) CaP orally daily for 14 days beginning 7 days before PC injection.

After animals in groups B to E had received drug treatment for 7 days they were anaesthetised with halothane/nitrous oxide/oxygen (2:1:2; flow rates for O₂ and N₂O = 1 and 2 litres/minute respectively). Each animal was under anaesthetic for at least 5 minutes before intra-articular injection and for a total of 10 minutes thereafter. Animals were given a single intra-articular injection of a preformed insoluble complex (PC) of poly-D-lysine (7.5 mg) and hyaluronan (HA) (7.5 mg) in 1 mL sterile, pyrogen-free isotonic saline in one knee. This preparation was prepared immediately prior to injection by quickly mixing 0.5mL sterile 15mg HA/mL with 0.5mL sterile poly-D-

lysine/mL in a 2mL sterile syringe. The contralateral knee joint of each animal was injected with 1 mL sterile isotonic saline only to serve as an internal control. Animals in groups B to E continued to receive daily oral drug preparations for further 7 days post PC injection.

- 5 Seven days post arthritis induction, rabbits were anaesthetised with halothane/nitrous oxide/oxygen as before and blood (20 - 40 mL) was obtained directly from the heart using a sterile 14 gauge catheter and a 50 mL syringe. 10 mL of this blood was deposited into sterile glass EDTA (K₃) vacutainer tubes with a final dilution of 0.7 % EDTA. A further 9mL was added to 1mL 5%(w/v) trisodium citrate and the
10 remainder was added to serum tubes. Within 2 hours of blood collection the separation of mononuclear cells and neutrophils was performed using the centrifugation procedure as described below. All blood samples were kept at room temperature at all times.

Separation and determination of blood white cells numbers

- 15 The EDTA-containing blood collected as described above was used to prepare white cell fractions. 20 mL of Percoll (AMRAD Pharmacia, Boronia, Melbourne Australia) at a density range between 1.070-1.072 g/mL, was prepared in sterile 40 mL Sorvall centrifuge tubes. This optimum density was achieved by first adding 9 parts of neat Percoll to 1 part of sterile 1.5M NaCl. This stock solution was then diluted using
20 0.15M NaCl until the desired density was obtained.

Centrifugation of Percoll and blood

- The Percoll, at an optimum density range of 1.070-1.072 g/mL, was centrifuged at 10,000 x g for 20 minutes at room temperature, using a fixed angle rotor head in a
25 Sorvall RC-5 centrifuge to form a gel which provides better separation of the white cells. 4 mL of the collected blood was then layered on top of the preformed density gradient and centrifuged at 1000 x g for 20 minutes at room temperature using a swinging bucket rotor in a Beckman TJ-6 centrifuge. From each of the density vials 1-7 (AMRAD, Pharmacia) 40 µL was added to 3720 µL of sterile 0.15M NaCl to give a
30 total volume of 4 mL. Each AMRAD vial contained coloured density marker beads of a given density. The density range of the 7 vials was 1.017-1.102 g/mL. The 4 mL density bead solution was then layered on top of a 20 mL preformed Percoll density gradient (1.07-1.072 g/ml), and centrifuged at 1000 x g for 20 minutes at room temperature with the sample tubes prepared above, using a swinging bucket rotor in a
35 Beckman TJ-6 centrifuge. This density marker tube served to calibrate the gradients

formed in the sample tubes. The following density ranges were used for the rabbit's blood (these differ from the manufacturer's instruction for human blood):

1. Mononuclear cells: 1.04-1.06 g/mL
2. Neutrophils: 1.06- 1.08 g/mL
- 5 3. Erythrocytes: 1.08-1.09 g/mL

After centrifugation of each sample tube, mononuclear cells and neutrophils were separated by aspiration at their designated density range and placed into separate centrifuge tubes. These cells were washed twice with 3 volumes of 0.15M NaCl at 10 1000 x g for 10 minutes at room temperature using a swinging bucket rotor in a Beckman TJ-6 centrifuge. The cell pellets of the mononuclear cells and neutrophils were resuspended with 500 μ L PBS. From each cell suspension 50 μ L was added to 50 μ L of trypan blue solution and the cell numbers quantitated using a haemocytometer. From the mononuclear cells another 50 μ L aliquot was used to form differential 15 slides. This procedure allowed the percentage of neutrophils, lymphocytes and monocytes to be determined. Smears were formed using a Cytospin centrifuge.

Dissection and collection of synovial membrane from joints for cell culture

Joints were sprayed liberally with 70% (v/v) ethanol and placed in a clean 20 plastic bag at 4°C for transport to the sterile environment of a laminar flow cabinet. Using rat-toothed forceps and a no. 22 scalpel blade, the tendons and excess muscle tissue were removed from the joints without opening the capsule.

Using small rat-toothed forceps and no.11 scalpel blade the joint capsule was opened aseptically using a posterior approach, severing the posterior insertions of the 25 cruciate ligaments to allow dis-articulation. The patella and the attached suprapatella mound of synovium was removed whole using a no. 11 blade. This tissue was then placed in a labelled specimen container filled with 10%(v/v) neutral buffered formaldehyde for histological processing. The remaining synovia was then harvested by excising pieces of the joint lining with the small rat-toothed forceps and dissection 30 with a no.11 blade, avoiding inclusion of excessive subsynovial tissue. Collected pieces were then floated into a labelled petridish containing 10mL sterile PBS.

Synovial fibroblast isolation

The synovia in the petridish was diced finely then transferred to a 15 mL sterile 35 centrifuge tube, using a sterile plastic transfer pipette with the fine end cut off (using a sterile no. 22 blade). The tubes were centrifuged at 1500 - 2000 rpm at 20°C for

10 minutes and the cell pellet resuspended in 5 mL sterile T&E [0.2% (w/v) trypsin/
0.1% (w/v) EDTA in PBS]. The tubes were placed at 4°C overnight (cold
trypsinisation) then were incubated at 37°C for one hour. After incubation 5 mL
DMEM/10% FCS was added (to inactivate the trypsin) and then the tubes were
5 recentrifuged at 20°C, 2000 rpm for 10 minutes and trypsin/FCS solution between the
pellet and floating fat layer was removed. Sterile DMEM/10% FCS was added to a total
volume of 10 mL and pellet was resuspended. The tubes were recentrifuged at 20°C,
2000 rpm for 10 minutes. The DMEM/10% FCS solution was removed and the pellet
was resuspended in 2 mg/mL collagenase in DMEM/10%FCS. The tubes were placed
10 in the incubator at 37°C for 3 hours then the collagenase solution, including fat layer,
was removed to waste. Sediment pellet was resuspended in 10mL DMEM/10%FCS
using a sterile plastic transfer pipette. Two x 5mL aliquots of the cell suspension were
placed into 2 x 25 cm² tissue culture flasks and incubated at 37°C. Media routinely
was changed every two or three days. When one of the flasks was ~90% confluent it
15 was used to determine HA synthesis. When the other flask was confluent, the cells
were subcultured.

In vitro HA biosynthesis by synovial fibroblast

Synovial fibroblasts as prepared above were plated into 6 well plates at 200,000 cells
20 per well and allowed to attach for 24 hours. To each well was added 2mL
DMEM+10%(v/v) FBS followed by 3H-glucosamine (stock is 1μCi per microlitre)
and plates incubated for 24 hours. After which, flasks were removed from the
incubator and chilled at 4°C and media was removed to labelled 15mL centrifuge tubes.
As media was removed, each flask was twice gently washed with 0.5mL nonsterile
25 PBS twice, which was added to the same labelled 15mL centrifuge tube. After the PBS
rinses, 1mL T+E was added directly to the cell layer in each well. The plate was
incubated at 37°C for 10 minutes. The released cell suspensions were transferred to
labelled 5mL tubes, rinsing the flasks twice with 0.5mL papain buffer and adding the
rinses to the same tubes.

30

Determination of radiolabelled HA synthesised by cells

Two x 0.5mL aliquots of each media sample were labelled A and B. To all
aliquots were added 20μL 1M acetic acid (to adjust pH to approx. 4.0). To each sample
labelled A was added 50μL 20mM sodium acetate/0.15M NaCl pH 6.0. To each sample
35 labelled B was added 50μL 20mM sodium acetate/0.15M NaCl pH 6.0 containing 5
TRU Streptomyces hyaluronidase. (Sigma Chemical Co). All samples were incubated

at 60°C for 3 hours the boiled for 5 minutes to inactivate the Streptomyces hyaluronidase. Samples were then be stored at -20°C prior to Superose 6 gel chromatography and determination of incorporated radioactivity into HA as already described above for the synthesis of HA by human synovial fibroblasts.

5

Immunohistology of Synovial Tissue

The patellae and suprapatella mound of synovium was removed whole from joints using the dissection procedure described above and the patellae and adhering synovium (and any pannus) sliced in half and each half placed in a specimen container
10 with neutral buffered formalin. Processing and preparation of stained and unstained tissue sections were performed as already described above

Immunolocalisation of Inducible Nitric Oxide Synthase (iNOS)

Four-micron patellae/synovium sections were deparaffinised to water.
15 Endogenous peroxidase was blocked with 3% H₂O₂ for 5 minutes. A high temperature heat retrieval was used to unmask antigenic sites using 10mM citrate buffer; pH 6.0. Non-specific binding was blocked with a serum-free protein block for 10 minutes. Sections were then incubated with the primary antibody polyclonal antiserum for iNOS (Cayman Chemicals Inc) 1:1000 overnight at 4°C. The negative control reagent used
20 was a protein concentration matched non-immune rabbit serum. The detection system utilised a commercial avidin-biotin with horseradish peroxidase as an enzyme label (Dako Biologics Pty Ltd). The secondary antibody cocktail of biotinylated goat anti-rabbit and biotinylated goat anti-mouse (Dako Biologics Pty Ltd) was applied for 15 minutes at room temperature. Slides were then incubated for 15 minutes in a
25 streptavidin conjugated to peroxidase (Dako Biologics Pty Ltd). Peroxidase activity was detected using 3,3'-diaminobenzidine (DAB). Sections were counterstained with Mayer's haematoxylin for 1 minute, dehydrated, cleared in xylene and mounted in Euckitt..

Immunolocalisation of Nitro-Tyrosine

The method used was identical to that described by Kobayashi *et al* (2001) for rabbit cartilages in another arthritis model(Kobayashi K, *et al.*, Chondrocyte apoptosis and regional differential expression of nitric oxide in the medial meniscus following partial meniscectomy. J Orthopaedic Res, 2001,19: 802-808)

35

Statistical methods

Differences between PC and saline injected joint data were assessed using paired t-tests. Non-treated, drug treated and non-drug treated PC or saline joint data were compared on each day using unpaired t-tests. Histological scoring (nonparametric data) was analysed using Wilcoxon signed rank tests. Analyses were performed using Microsoft Excel 98 or Statview 5.0 on an Apple Macintosh PowerPC. P values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

The present inventors have made the unexpected and surprising discovery that subjecting cartilage particles to autolysis in aqueous buffers maintained within the pH range of 4.0-7.0, particularly 4.5, at 37°C for periods up to 36 hours, particularly 16 or 24 hours specifically released GAG-peptide complexes and matrix derived polypeptides into solution while leaving the tissue cells and their intracellular macromolecular components essentially in tact. In this context we used DNA as a marker for intracellular macromolecular components and demonstrated that the autolysis medium obtained by the present invention was substantially free of DNA. Moreover, these released GAG-peptide complexes, defined arbitrarily as Peptacans when used alone or in combination with other co-released polypeptides are pharmacologically more active than commercially available ChS.

The release of these cartilage products was found to be more rapid with smaller particles than larger ones (FIGURE 1). However, when either cartilage was incubated at 37°C over a 24 hour period 75-83% of the total tissue sulfated GAGs was released into solution as shown by analysis for this component irrespective of the particle size (FIGURE 1).

The efficiency and selectivity of this method was also confirmed by histochemical analysis of the cartilage particles before and after subjecting them to the inventive method. FIGURE 2 shows photomicrographs of histological sections of 3mm bovine tracheal cartilage powders particles before and after subjecting them to the autolysis procedure using 100mM calcium acetate buffer, pH 4.5 at 37 degrees C. Panels A and C are sections of cartilage before autolytic processing and B and D after. Panels A and B show the results of staining with Toluidine Blue (TB), a dye which binds to glycosaminoglycans (GAGs) while C and D show sections stained with Masson Trichrome (Masson TC), a dye known to stain native collagen fibres magenta colour. Note the loss of staining for GAGs in section B after autolysis but a slightly increased intensity of staining for collagen following the removal of the majority of

GAGs as shown in D. Cell nuclei, identified in these sections by the green counter-stains are clearly unchanged by the autolytic process. All sections are shown at magnification X 400.

As is evident from FIGURE 2 the level of staining for the presence of sulfated
5 GAGs in cartilage particles using the cationic stain for these molecules, Toluidine Blue, before processing was extensive but after incubation was diminished. On the other hand, Masson Trichrome staining of native collagen fibres was essentially unchanged by the autolytic process (FIGURE 2). Significantly, the cells and importantly their nuclei which were clearly visible using both staining procedures of the residual
10 cartilage, remaining after removing the supernatant and washing with the incubation buffer, were observed to be largely undisturbed (FIGURE 2).

The autolytic process was affected by both pH and temperature. As is evident from FIGURE 3 the most rapid release of peptacans from bovine tracheal cartilage occurred within the pH range 4.0 - 7.0. The normal mammalian blood temperature of
15 37 degrees C was found to be more effective than lower temperatures, most notably 4°C in facilitating peptacan release while intermediate levels of release occurred at 25 degrees C (FIGURE 4). The influence on this release process of both temperature and pH was consistent with the notion that autolysis was proceeding via the cleavage of matrix proteoglycans and other structural proteins by endogenous enzymes whose
20 catalytic activities were optimum within the pH range 4.0-7.0 at 37 degrees C. This explanation was confirmed by the observation that the release process was substantially slowed by maintaining the cartilages particles in the autolysis buffers at 4°C as well as the observation that the rate of release was markedly reduced by including specific inhibitors of proteinases in the incubations in buffers at 37 degrees C (FIGURE 5). As
25 is evident from FIGURE 5, approximately 50% inhibition of the autolytic process was achieved by the addition of the cysteine proteinase inhibitor, N-ethylmaleimide to the buffer solution. Since the major cysteine proteinases of cartilage are the Cathepsins (Dingle JT, The secretion of enzymes into the pericellular environment. Phil Trans Royal Soc (London), 1975, 271: 315-324; Barrett AJ and McDonald JK, Mammalian
30 Proteases, Academic Press, London, New York, 1980, pp338-350; Barrett AJ *et al.*, Lysosomal cysteine proteinases, ISC Atlas Sci Biochemistry, 1988,1: 256-260, Muller-Ladner U, Gay RE, Gay S, Cysteine proteinases in arthritis and inflammation, Perspectives in Drug Discovery and Design, 1996, 6:87-98) which have pH optima between 3.5 - 6.0, it is most likely that these enzymes are largely responsible for the
35 autolytic degradation of matrix components in this invention. However, as matrix component degradation was not completely abrogated by N-ethylmaleimide other

classes of proteinases are clearly involved. Since the serine proteinase inhibitor, Benzamidine had no observable effect on release, this class of proteinases would seem to be excluded. However, the matrix metalloproteinases (Birkedal Hansen H, *et al.*, Matrix metalloproteinases: a review. Critical Rev Oral Bio Med, 1993, 197: 197-250) and
5 aggrecanases (Ilic MZC, Handley CJ, Robinson HC, Mok MT, Mechanism of catabolism of aggrecan by articular cartilage, Arch Biochem Biophys, 1992, 294:115-122) which have previously been shown to degrade cartilage matrix components at neutral pH are potential candidates.

As already discussed herein the matrix degradation products generated by these
10 endogenous enzymes are structurally different from those produced by digesting connective tissue with papain or proteinases of other origins. Since the pharmacological activity of drugs and biopharmaceuticals is critically dependent on their composition and structure, we consider that the peptacans and the other autolytic products generated by the present inventive method cannot be reproduced by simply
15 digesting cartilages with exogenous enzymes such as papain, chymopapain, bromelain, pepsin, trypsin, chymotrypsin, elastase or other enzymes which are not specifically derived from the tissues themselves.

The efficiency of the autolytic process in the present invention was also influenced by the animal species and anatomical location from where the cartilage
20 was derived. As is evident from TABLE 1 below, bovine tracheal and nasal cartilages afforded the highest yields of peptacans when incubated at 37°C over 24 hours. However, deer antler cartilages and avian (chicken) sterna provided more than 60% yield of peptacans under the same conditions. Surprisingly, commercially available samples of shark cartilage, of unknown location or method of preparation afforded very
25 low yields of peptacans. Nevertheless, it is clear from these examples that the method of the invention is applicable to a wide range of connective tissues but the efficiency of release of peptacans into the aqueous medium is related to their ultrastructural assembly and means of preparation prior to autolytic treatment.

TABLE I

Kinetics of sulfated glycosaminoglycan (GAG) release from cartilages of various origins over 24 hours using 100mM Calcium Acetate buffer pH 4.5.

Time (hr)	% GAG released						
	(B-Tracheal)	(A-Sternea)	(B-Nasal)	(Shark)	(O-Articular)	(O-Meniscus)	(C-Antler- tip) (C-Antler- mid)
0	0.54	2.91	0.08	6.67	1.13	0.00	0.00
1	13.26	41.55	10.64	30.50	3.38	0.00	5.18
2	28.96	41.97	28.86	30.73	7.22	0.00	19.42
3	45.36	46.26	42.09	27.28	13.96	0.06	42.40
4	60.75	48.75	50.51	24.95	17.97	0.10	56.63
5	65.61	45.71	52.14	24.95	18.57	0.38	56.45
24	93.16	60.90	81.36	23.65	51.03	0.52	56.31

B = Bovine, A = Avian, O = Ovine, C = Cervine, Antler-mid = cartilage between tip and bone, Shark = unknown site.

When the supernatants derived from the tracheal cartilage incubated with 0.1M Calcium Acetate were applied to a Superdex gel filtration column and fractions monitored for sulfated-GAGs and protein two resolved peaks corresponding to the GAG-peptide and polypeptides were obtained (FIGURE 6). Evidence that the GAG-peptide was not simply ChS but contained more than one sulfated GAG chain was provided by its digestion with the proteolytic enzyme papain which shifted the DMMB positive peak to the same column fractions obtained when pure ChS was chromatographed under the same conditions (FIGURE 7). When the supernatants obtained from incubation of 0.1M Calcium acetate pH 4.5 (CaP) and dilute acetic acid pH 4.5 (H2OP) were chromatographed under the same conditions on the Superdex-200 column it was observed that the GAG-peptide obtained with the Calcium Acetate buffer was of smaller molecular size than that released by the acetic acid digestions. Both were, however larger than two commercially available ChSs (FIGURE 8). The weight average molecular masses of the CaP, H2OP, purified CaP (pCaP) and the two commercially available ChSs were then assessed using a Sephadex-G 200 high resolution gel exclusion column and seven ChS standards prepared and characterised previously (Melrose J and Ghosh P, Determination of the average molecular size of glycosaminoglycans by fast protein liquid chromatography. J Chromatography, 1993, 637; 91-95). The correlation between MW and Kav obtained using these ChS standards and the Sephadex column is shown in FIGURE 9 and the results obtained for the unknown preparations is shown in TABLE 2 below. These experiments confirmed that the GAG-peptide in CaP contained 2 ChS chains while that in H2OP consisted of 3 ChS chains.

25

TABLE 2

Average Molecular Size (KDa) of commercial chondroitin sulfates and Peptacans as determined by Gel Chromatography and standards

ChSA (Sigma)	17.3
ChSA (Bioiberica)	20.0
CaP	33.7
pCaP	31.1
H2OP	46.1
MgP	32.3
ZnP	50.4

30

CAPAGE of CaP, pCaP and papain digested CaP indicated that the only sulfated GAG present was ChS (FIGURE 10). However, SDS-PAGE showed that CaP also

contained a number of peptides with MWs of 21 KDa or less which were not present in the purified CaP (FIGURE 11). Figure 11 shows analysis of proteins and peptides in Peptacans Using SDS-Polyacrylamide gel electrophoresis. Lane 1= protein markers with molecular weights shown, lane 2 = CaP, lane 3 = purified CaP, lane 4 = chondroitinase-5 ABC digested and purified CaP.

Analysis for total protein and hydroxy proline content as a marker of collagen of these samples suggested that the dominant polypeptides released were largely derived from cleavage of collagen chains.

10

TABLE 3

Analysis of Peptacans and Commercial Chondroitin Sulfates For Sulfated Glycosaminoglycans, Protein and DNA content

Samples	S-GAG(DMMB) %	Protein (BCA) %	DNA(Hoechst) %
ChSA (Sigma)	98.5	0.16 ^a	0.36
ChSA (Bb 1/0015,05/2001)	97.4	1.25 ^a	0.32
ChSA (Bb 18/11/99)	98.1	0.63 ^a 0.74 ^a	0.35 UN
ChSA (NaP)	97.8		
ChSA (CaP)	96.5	1.40 ^a	UN
CaP	39.8	35.12 ^b	UN
pCaP (EtOH ppt)	49.0	40.51 ^b	UN
H2OP	65.6	44.48 ^b	UN
NaP	53.3	32.93 ^b	UN
pH2OP (column)	85.0	9.60 ^b	UN
pCaP (column)	44.8	6.52 ^b	UN
MgP	32.5	36.59 ^b	UN
ZnP	18.6	38.17 ^b	UN

15

S-GAGs = sulfated glycosaminoglycans (method of assay used, see text)
UN = undetectable, a = using BSA as standard , b = using Gelatin as standard

Spectroscopic analysis of concentrated aqueous solutions (1.0mg/ml) of the Peptacans (CaP, NaP, H2OP) and commercial ChSs (FIGURE 12) and ChSs derived from Peptacans revealed low absorption for protein at 280nm and DNA at 260nm for the Peptacans and the ChSs derived from them. In contrast high values for DNA were evident in the commercial ChS preparations as indicated by the ratios of A260/A280 in their uv spectra which were in the order of 1.85 or more (FIGURE 12). Using a published fluorescent dye binding assay for DNA in the same samples and calf thymus DNA as a standard confirmed that the Peptacan preparations and the ChSs derived from

25

them were substantially free of DNA, whereas the pharmaceutical quality commercial ChS examined contained approximately 0.3% DNA (TABLE 3). Analysis of the relative binding of CaP and ChS to the enzymes, lysozyme, hyaluronidase and human neutrophil elastase which are mediators of matrix destruction in arthritis using the

5 BIAcore 2000 system showed that CaP interacted more strongly with lysozyme and elastase than ChS but with hyaluronidase there was little difference between the two preparations (FIGURE 13). The higher binding affinity of CaP for the proteolytic enzyme, elastase derived from human neutrophils was confirmed using a conventional

10 functional assay and the synthetic elastase substrate, succinyl-alanine-alanine-valine-nitroanilide (SAAVN). Using this assay system the concentration range of ChS which produced 50% inhibition of elastase (IC50) was found to be 4-5 micrograms/ml while the IC50 for the CaP preparation was between 1-3 micrograms/ml.

In the short term (2 days) chondrocyte cultures CaP at concentrations of 250 micrograms/ml and above stimulated cell division as indicated by tritiated thymidine

15 incorporation into DNA while ChS had no effect (FIGURE 14). In the longer term chondrocyte cultures (7 days) CaP at 62.5 micrograms/ml stimulated DNA synthesis (FIGURE 15). These data clearly identify the anabolic effects of the CaP which would support the repair and regeneration of connective tissues.

As already indicated PGs are essential components of the cartilage extracellular

20 matrix and are responsible for its unique biomechanical properties. Since these molecules are degraded and depleted from cartilage in arthritic joints, agents which can prevent this event or stimulate their biosynthesis by chondrocytes and other cells would be beneficial to the recovery of joint function and a reduction in patient symptoms.

In contrast to ChS, CaP exhibited a concentration dependent stimulation of

25 proteoglycan synthesis by chondrocytes with more than 150% deposition of PGs into the extracellular matrix at CaP concentrations of 1.0 and 2.0 mg/ml (FIGURE 16). At concentrations between 62.5 – 500 micrograms/ml ChS released slightly more PGs into the media than CaP but this was reversed at 2.0mg/ml. (FIGURE 16). Significantly, when the effects of CaP and ChS on PG synthesis by chondrocytes was studied in

30 cultures to which the proinflammatory cytokine interleukin-1 (IL-1) had been added. CaP produced a 500% increase in synthesis at concentrations of 1.0mg/ml while ChS had no effect (FIGURE 17). Interestingly this stimulatory effect by CaP on PG synthesis was greater than when cells were cultured in the absence of IL-1 (FIGURE 17).

35 The knee joint menisci are structurally complex fibrocartilages which perform an essential role for the weight bearing functions of diarthrodial joints. They are

crescent shaped cartilages in which the outer rim is under high tensile stresses and the inner region is subjected to more compressional loading. The fibrochondrocytes from these two regions are therefore metabolically different and were for this reason studied separately. When fibrochondrocytes from the inner region of ovine menisci were
5 cultured with various concentrations of ChS and the Peptacans, only CaP stimulated PG synthesis (FIGURES 18 &19). At 2.0mg/ml a 200% stimulation of PG synthesis was obtained for CaP and a 130% increase for ChS but only for PGs released into the media (FIGURES 20 &21). CaP was observed to produce a similar PG stimulatory profile on
10 fibrochondrocytes isolated from the outer region of the meniscus (FIGURES 22,23 & 24) but in these cultures ChS failed to elicit a significant increase in media PG levels (FIGURE 25).

The major non-proteinaceous component of joint synovial fluid is hyaluronan (HA). As already indicated HA confers to synovial fluid its unique rheological properties which includes exceptionally efficient lubrication of articular cartilage and
15 peri-articular tissues. In rheumatoid and osteoarthritic joints synovial fluid HA concentration and molecular weight are decreased (Dahl LB, Dahl IMS, Engstrom-Laurent A, Granath K. Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. *Ann Rheum Dis* 1985;44:817-22). Since the rheological effects of HA are dependent on its
20 molecular weight and concentration, a decline in either or both of these parameters decreases the ability of synovial fluid to efficiently lubricate and protect articulating surfaces. However, HA is also anti-inflammatory and exhibits a number of other important functions essential in joint physiology (Ghosh P and Guidolin D, Potential
25 mechanism of action of intra-articular hyaluronan therapy in osteoarthritis: Are the effects molecular weight dependent?, *Seminars in Arthritis and Rheumatism* 2002, 32: 10-37.). Diminished HA concentration and a reduction in its molecular weight could therefore have profound effects, not only on the rheological properties of synovial
30 fluid, but synovial and inflammatory cell functions and their macromolecular expression. Synovial fluid HA is almost exclusively synthesized by type B cells of the synovial lining. These fibroblasts (synoviocytes) when isolated from synovial joints and established in culture retain their phenotypic expression and elaborate HA into culture media. Fibroblast cultures derived from rheumatoid or osteoarthritic joints show
aberrant biosynthesis of HA reflecting the abnormal metabolic status of the joints from which they were derived (Smith MM, Ghosh P. The synthesis of hyaluronic acid by
35 human synovial fibroblasts is influenced by the nature of the hyaluronate in the extracellular environment. *Rheumatol Int* 1987;7:113-22). Generally the amount of HA

synthesized is less than obtained from normal synoviocytes and its molecular weight is reduced.

In the present experiments Peptacans and ChS were evaluated for their respective abilities to promote the biosynthesis of DNA and high molecular weight HA by synovial fibroblasts obtained from joints of patients with OA. The results obtained are shown in FIGURES 26-29. As is evident from FIGURE 26 only CaP exhibited a significant effect on DNA synthesis at concentrations up to 250 micrograms/mL. However, HA synthesis by these cells was increased 230% at 250 micrograms/mL (FIGURE 27) and 380% at 2.0mg/mL (FIGURE 28). The identity molecular weight of the HA synthesised was confirmed by chromatography (FIGURE 27) and agarose gel electrophoresis (results not shown). The relative activities of ChS, CaP and H2OP when incubated with the cells at a concentration of 2.0mg/ml are shown in FIGURE 28. The reason for the significant differential effects between CaP and H2OP on HA synthesis by these cells is presently unresolved but was a surprising finding.

The relative activities of CaP, pCaP and pH2OP studied under the same conditions are included in FIGURE 29. This result shows the higher activity of CaP relative to the column purified (pCaP) preparation with regard to the stimulation of HA synthesis by human OA synovial fibroblasts. This result suggests that polypeptides in CaP may be acting synergistically with the calcium GAG-peptide complex.

The in vitro stimulation of the biosynthesis of HA by synovial fibroblasts derived from joints of humans with osteoarthritis by CaP was also demonstrated to occur in vivo using an animal model of arthritis. In these latter experiments ChS at 300mg/kg or CaP at 100, 200, or 300mg/kg was administered orally to rabbits in which monoarticular proliferative arthritis had been induced by the intra-articular injection of a PC-complex. The preparations were given daily for 7 days before arthritis was induced and daily for 7 days thereafter. Animals were euthanased and blood and tissues collected for analysis on day 14. Synovial fibroblasts from the joints of these animals together with those from a non-drug treated arthritis control group were established in primary cell culture and the biosynthesis of radiolabelled HA determined as described for the in vitro studies. The results of these experiments are shown in FIGURES 30 and 31. Compared to the non-drug treated control group synovial fibroblasts from all animals in the CaP treated group showed higher synthesis of HA (FIGURE 30), however, because of the inter-animal biological variation only the group receiving the dose of 300mg/kg were statistically significant ($p < 0.05$) relative to the non-drug-treated group (FIGURE 31). If the comparison of HA stimulation by the treatments was based on their respective sulfated GAG content then CaP was more

than 3X more active than ChS. Promotion of HA synthesis by synovial cells in arthritic joints would contribute to improved joint lubrication and a reduction of cartilage degradation as has already been discussed in detail elsewhere in this disclosure.

The extensive synovial inflammation provoked in the rabbit joints by the intra-
5 articular injection of the arthritogen (PC complex) was very apparent from the histological sections of the synovial membranes which showed massive cellular infiltration, connective proliferation, hyperplasia and neovascularization. In the non-drug treated control group these inflammatory events were further characterised by the presence of an invasive pannus originating from the synovial tissues at the joint
10 margins. Beneath and adjacent to this pannus tissue destruction of cartilage and subchondral bone was evident (FIGURES 32 and 33). In the drug treated groups synovitis was not abrogated but the extent of pannus formation was reduced and the structural integrity of cartilage largely preserved. Furthermore, the expression by chondrocytes in the patella cartilage of inducible nitric oxide synthase (iNOS), the
15 intra-cellular enzyme responsible for the production of nitric oxide free radicals, was mitigated particularly in the high dose CaP treated animals (FIGURE 32). This observation was consistent with the level of immunohistochemical staining for nitrotyrosine, which is a characteristic product marker of the chemical action of nitric oxide radical on intra-cellular proteins (Kobayashi K, *et al.*, Chondrocyte apoptosis
20 and regional differential expression of nitric oxide in the medial meniscus following partial meniscectomy. J Orthopaedic Res, 2001,19: 802-808). As can be seen in Figure 33 staining for this product was substantially reduced in chondrocytes from cartilages from joints of the CaP treated animals . This reduction in chondrocyte production of iNOS and thus nitric oxide free radicals by CaP treatment is considered to arise from its
25 ability to modulate the effects of pro-inflammatory cytokines (such as interleukin -1) as suggested by the in-vitro experiments. Interleukin -1 is known to be a major initiators of nitric oxide and other free radical production, synovitis inflammation and tissue destruction within arthritic joints (Taskiran D, Stefanovic-Racic M, Georgescu H, Evans C. Nitric oxide mediates suppression of cartilage proteoglycan synthesis by
30 interleukin-1. Biochem Biophys Res Commun 1994;200:142-8). However, CaP could also have a direct inhibitory effect on nitric oxide radical activity or its production by synovial cells and chondrocytes which in itself would be a useful means of suppressing inflammation and tissue destruction in arthritic joints . Such a strategy which has already been suggested by others using synthetic iNOS inhibitors (US Patent
35 6,346,519, February 2002).

In addition to the beneficial effects mediated by CaP on the joint tissues of the rabbit arthritis model described above, it was also found to suppress the levels of both neutrophils and mononuclear cells circulating in the blood of the arthritic animals (FIGURES 34 and 35). Using the ChS treated animals as drug treated control group, it was evident that this suppressive effect of CaP was dose dependent, the 300mg/kg dose being the more effective, particularly on circulating neutrophil numbers (FIGURE 34). While these blood borne white cells serve as an important vanguard in the immune defense against invading pathogens, once activated, as occurs in inflammatory states such as rheumatoid arthritis and osteoarthritis, emphysema, inflammatory bowel disease, vasculitis and suchlike, they provide an ongoing source of destructive proteinases, oxygen derived free radicals and pro-inflammatory cytokines all of which contribute to the initiation and perpetuation of the disease process. Immunosuppression in these inflammatory conditions, has and continues to be, an important therapeutic objective for their treatment (St. Georgiev V, Immunomodulating drugs: major advances in research and development. in Immunomodulating drugs, Editors : St. Georgiev V and Yamaguchi H, Ann NY Acad Science, 1993, 685, 1-10). Moreover, immunosuppressive therapy has been extensively used for the management of chronic rheumatoid arthritis for over 30 years (Weinblatt ME, Immunosuppressive therapy in rheumatoid arthritis, in: Therapeutic control of inflammatory disease, new approaches to antirheumatic drugs, Editors: Otterness I, Lewis A, Capetola R, Advances in inflammation research, Vol 11, Raven Press, New York, 1986, pp265-276). Although the suppressive effects on white cells observed for high oral dose CaP in the rabbit model of mono-articular proliferative arthritis used here appear to be a novel finding for a nutraceutical preparation, it is known that oral administration of cartilage derived antigens, such as type II collagen peptides can induce cell anergy and/or active cellular suppression of immune responses by promoting clonal selection and the secretion of anti-inflammatory cytokines, such as interleukins -4 and 10 and transforming growth factor- beta (TGF-beta). Furthermore, oral administration of these cartilage antigens have been used to treat inflammatory joint diseases such as rheumatoid arthritis (Kagnoff MF, Oral tolerance: mechanisms and possible role in inflammatory joint diseases, Baillieres's Clinical Rheumatology, 1996, 10: 41-54; Weiner HL and Komagata Y., Oral Tolerance and the treatment of rheumatoid arthritis. Sem Immunopathol, 1998, 20: 289-308)

INDUSTRIAL APPLICABILITY

GAG-peptide complexes and polypeptides substantially free of DNA may be used, either directly or after further processing, for the treatment, protection and restoration of connective tissues in inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and as anti-angiogenic agents for the treatment of cancers.

Because the compounds are substantially free of DNA, they are also substantially free of other molecules such as viruses that may be associated with DNA. An avoidance of such associated molecules could avoid health risks. Bovine and other animal nucleic acids are strongly bound to or form complexes with retroviruses and heat/protease resistant prion proteins which have been implicated in the spread of transmissible spongiform encephalopathies such as Creutzfeld-Jakob disease, kuru, Gerstmann-Straussler-Scheiner syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathies in cattle.

Calcium salts of GAG-peptide complexes have been found to have particular pharmacological efficacy which is further enhanced in combination with matrix polypeptides. Ingestion of calcium salts would provide an added benefit in being a source of dietary calcium.

The method of the present invention is essentially non-disruptive to the connective tissue used. Thus the residual tissue particles remaining after the autolysis is completed and the medium containing the GAG-peptide complexes and polypeptides has been removed, can still be used as a source of collagen derivatives. In this regard it should be noted that traditional methods for preparing ChS from connective tissues such as cartilage use exhaustive proteolytic or chemical digestion to destroy the proteinaceous components of the matrix to allow the ChS to be released into aqueous solutions and isolated. The residual cartilage particles obtained by the present method can be hydrolysed proteolytically or chemically to obtain collagen peptides of variable molecular size to be used for the treatment, protection and restoration of connective tissues in inflammatory and degenerative disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms or for the enhancement of wound healing or for the preparation of artificial biomatrices for cell culture, cell transplantation, or delivery of bioactive compounds including drugs and growth factors into a host tissue.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly

described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

THE CLAIMS

1. A method for the preparation of a connective tissue-derived GAG-peptide complex and polypeptide said method comprising the steps of:
subjecting particles of connective tissue to enzyme mediated autolysis by contacting
5 with an autolysis medium containing a monovalent or divalent salt at an effective pH
and temperature such that at least one GAG-peptide complex and at least one
polypeptide are released substantially free of DNA, by the proteolytic actions of an
endogenous enzyme(s) from within the tissue particles, into the autolysis medium,
leaving residual tissue particles; and
10 recovering the GAG-peptide complex(es) and polypeptide(s) from the autolysis
medium.
2. A method according to claim 1 wherein the monovalent salt is selected from the
group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof.
3. A method according to claim 1 wherein the divalent salt is selected from the
15 group consisting of calcium, magnesium, copper, zinc and mixtures thereof, preferably
calcium and magnesium.
4. A method according to claim 1 wherein the pH is in the range of from 2.5 to 8.5,
preferably 3.5 to 8.0, more preferably 4 to 7, most preferably 4.5 to 7.
5. A method according to claim 1 wherein the temperature is in the range of from
20 20 to 45°C, preferably 25 to 45°C, more preferably 32 to 45 °C, most preferably 37°C.
6. A method according to claim 1 wherein the connective tissue is selected from
the group consisting of skin, bone, tendon, ligament and cartilage.
7. A method according to claim 1 wherein the connective tissue is selected from
the group consisting of:
25 bovine, ovine, porcine, cervine and equine, tracheal, articular, auricular and
nasal cartilage;
chicken sternal cartilage; chicken rib cartilage; shark skeletal cartilage; and
growing deer antler cartilage.
8. A method according to claim 7 wherein the connective tissue is selected from
30 the group consisting of growing deer antler cartilage, bovine tracheal, articular and
nasal cartilage, preferably bovine tracheal and nasal cartilage.
9. A GAG-peptide complex substantially free of DNA and/or polypeptide
substantially free of DNA obtainable by the method according to any one of claims 1 to
8.

10. A chondroitin sulfate salt derived from a GAG-peptide complex substantially free of DNA, said GAG-peptide complex being obtainable by the method according to any one of claims 1 to 8.

11. A monovalent or divalent salt of a GAG-peptide complex substantially free of
5 DNA wherein the monovalent salt is selected from the group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof, and the divalent salt is selected from the group consisting of calcium, magnesium, copper, zinc and mixtures thereof.

12. A monovalent or divalent salt of a polypeptide substantially free of DNA wherein the monovalent salt is selected from the group consisting of hydrogen, sodium,
10 potassium, ammonium and mixtures thereof, and the divalent salt is selected from the group consisting of calcium, magnesium, copper, zinc and mixtures thereof.

13. A monovalent or divalent salt of chondroitin sulfate substantially free of DNA wherein the monovalent salt is selected from the group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof, and the divalent salt is selected from the
15 group consisting of calcium, magnesium, copper, zinc and mixtures thereof.

14. Use of at least one GAG-peptide complex substantially free of DNA and/or chondroitin sulfate salt substantially free of DNA for the manufacture of a medicament for treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and
20 myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers.

15. Use according to claim 14 wherein the chondroitin sulfate salt is a monovalent or divalent salt and wherein the monovalent salt is selected from the group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof, and the divalent salt
25 is selected from the group consisting of calcium, magnesium, copper, zinc and mixtures thereof.

16. Use of at least one GAG-peptide complex substantially free of DNA and at least one polypeptide substantially free of DNA for the manufacture of a medicament for treatment of inflammatory and degenerative tissue disorders such as rheumatoid
30 arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers.

17. Use of a calcium salt of at least one GAG-peptide complex substantially free of DNA and/or a calcium salt of chondroitin sulfate substantially free of DNA for the
35 manufacture of a medicament for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms,

atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamis and for the treatment of cancers .

18. Use of a calcium salt of at least one GAG-peptide complex and a calcium salt of at least one polypeptide substantially free of DNA for the manufacture of a medicament
5 for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamis and for the treatment of cancers.

19. Use according to claim 18 wherein the calcium salt of the GAG-peptide
10 complex is substantially free of DNA.

20. Use according to claim 18 wherein the calcium salts of the GAG-peptide complex(es) and the polypeptide(s) are co-isolated from connective tissue, preferably tracheal cartilage, more preferably bovine tracheal cartilage by the method according to any one of claims 1 to 8.

21. Use of at least one polypeptide substantially free of DNA for the manufacture of a medicament for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms.

22. A method for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms,
20 atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamis and for the treatment of cancers comprising the administration to a subject in need of such treatment an effective amount of at least one GAG-peptide complex substantially free of DNA and/or chondroitin sulfate salt substantially free of DNA.

23. A method according to claim 22 wherein the chondroitin sulfate salt is a monovalent or divalent salt and wherein the monovalent salt is selected from the group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof, and the divalent salt is selected from the group consisting of calcium, magnesium, copper, zinc and mixtures thereof.

24. A method for the treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamis and for the treatment of cancers comprising the administration to a subject in need of such treatment an effective
35 amount of at least one GAG-peptide complex substantially free of DNA and at least one polypeptide substantially free of DNA.

25. A method of treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers comprising the administration to a subject in need of such treatment an effective amount of a calcium salt of at least one GAG-peptide complex substantially free of DNA and/or a calcium salt of chondroitin sulfate substantially free of DNA.
26. A method of treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms, atherosclerosis and myocardial ischemia, degenerative and diabetic arteriopathies, thrombosis and embolisms, hyperlipideamias and for the treatment of cancers comprising the administration to a subject in need of such treatment a calcium salt of at least one GAG-peptide complex and at least one polypeptide substantially free of DNA.
27. A method of treatment of inflammatory and degenerative tissue disorders such as rheumatoid arthritis and osteoarthritis in any of their multiple forms comprising the administration to a subject in need of such treatment an effective amount of at least one polypeptide substantially free of DNA.
28. A pharmaceutical composition comprising at least one GAG-peptide complex substantially free of DNA and/or chondroitin sulfate salt substantially free of DNA, together with a pharmaceutically acceptable carrier.
29. The pharmaceutical composition of claim 28 wherein the chondroitin sulfate salt is a monovalent or divalent salt and wherein the monovalent salt is selected from the group consisting of hydrogen, sodium, potassium, ammonium and mixtures thereof, and the divalent salt is selected from the group consisting of calcium, magnesium, copper, zinc and mixtures thereof.
30. A pharmaceutical composition comprising at least one polypeptide substantially free of DNA together with a pharmaceutically acceptable carrier.
31. A method according to claim 1 further including the step of hydrolysing the residual tissue particles to obtain collagen peptides.
32. Collagen peptides prepared by the method according to claim 31.

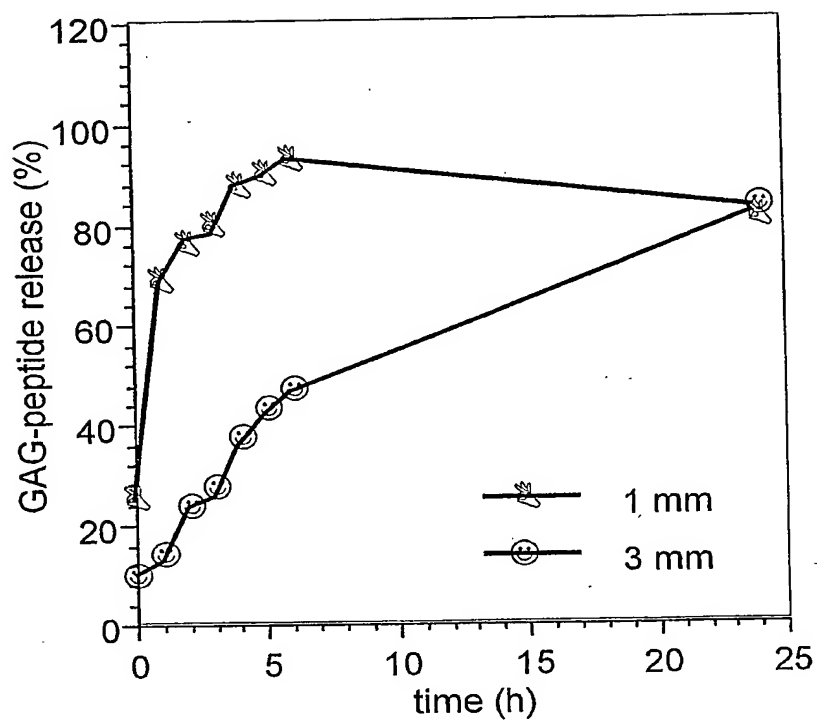
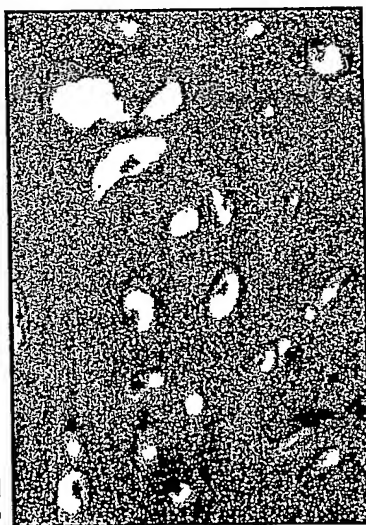
1/35
FIGURE 1

FIGURE 2

Bovine Tracheal Cartilage

- before [A & C] and after treatment [B & D]

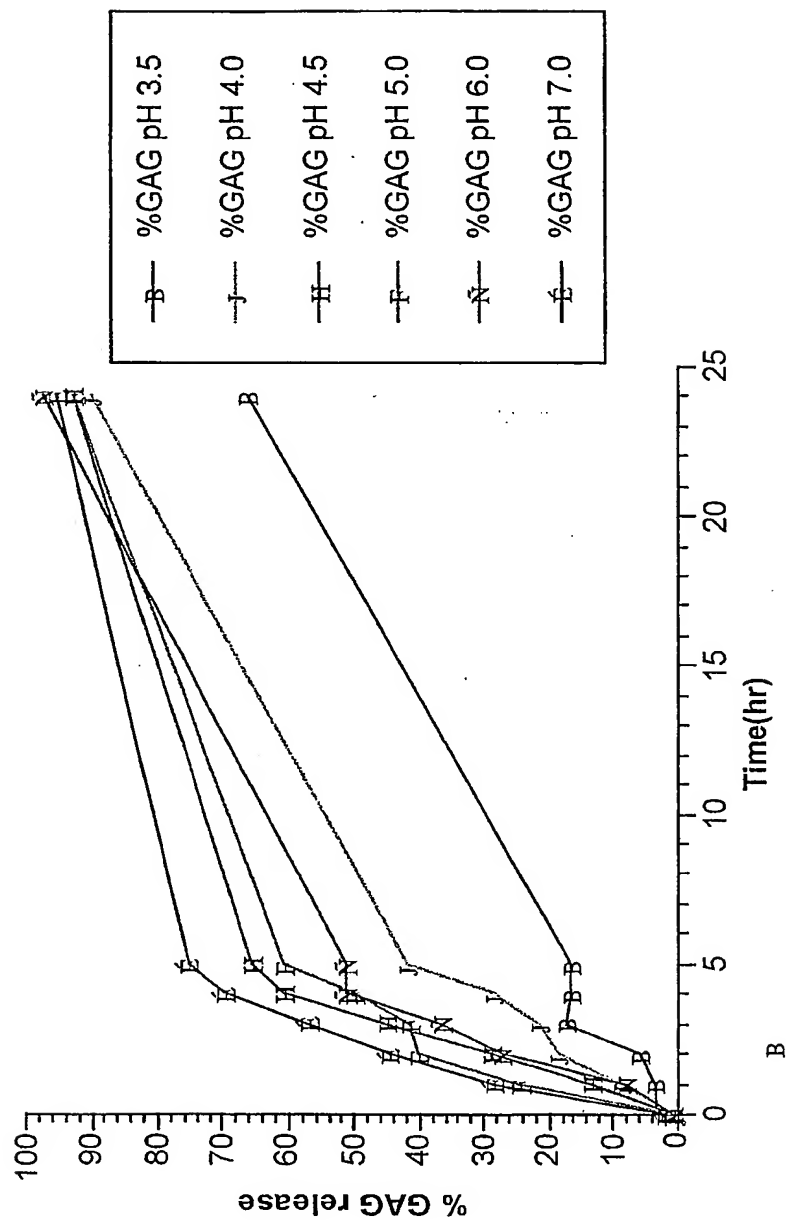
[A] TB stain = Sulfated GAGs [B] TB stain = Sulfated GAGs



[C] MassonTC stain = Collagen [D] MassonTC stain = Collagen

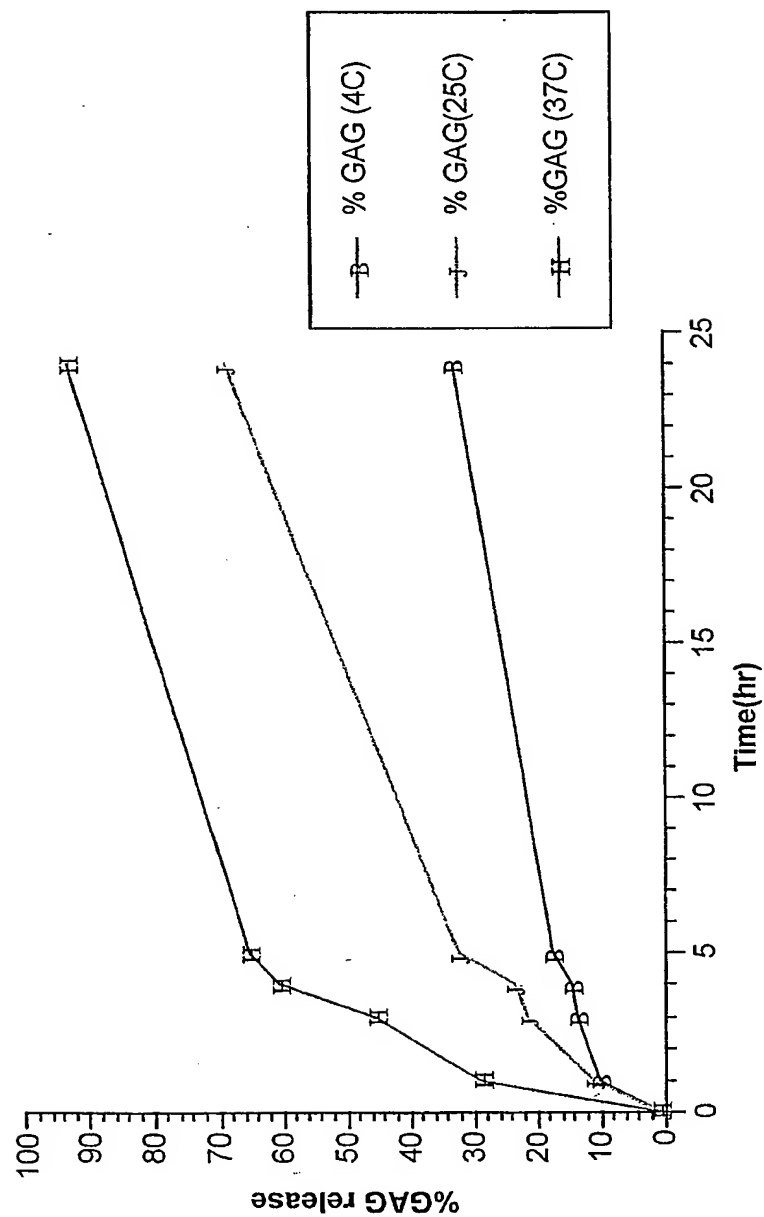


FIGURE 3



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FIGURE 4



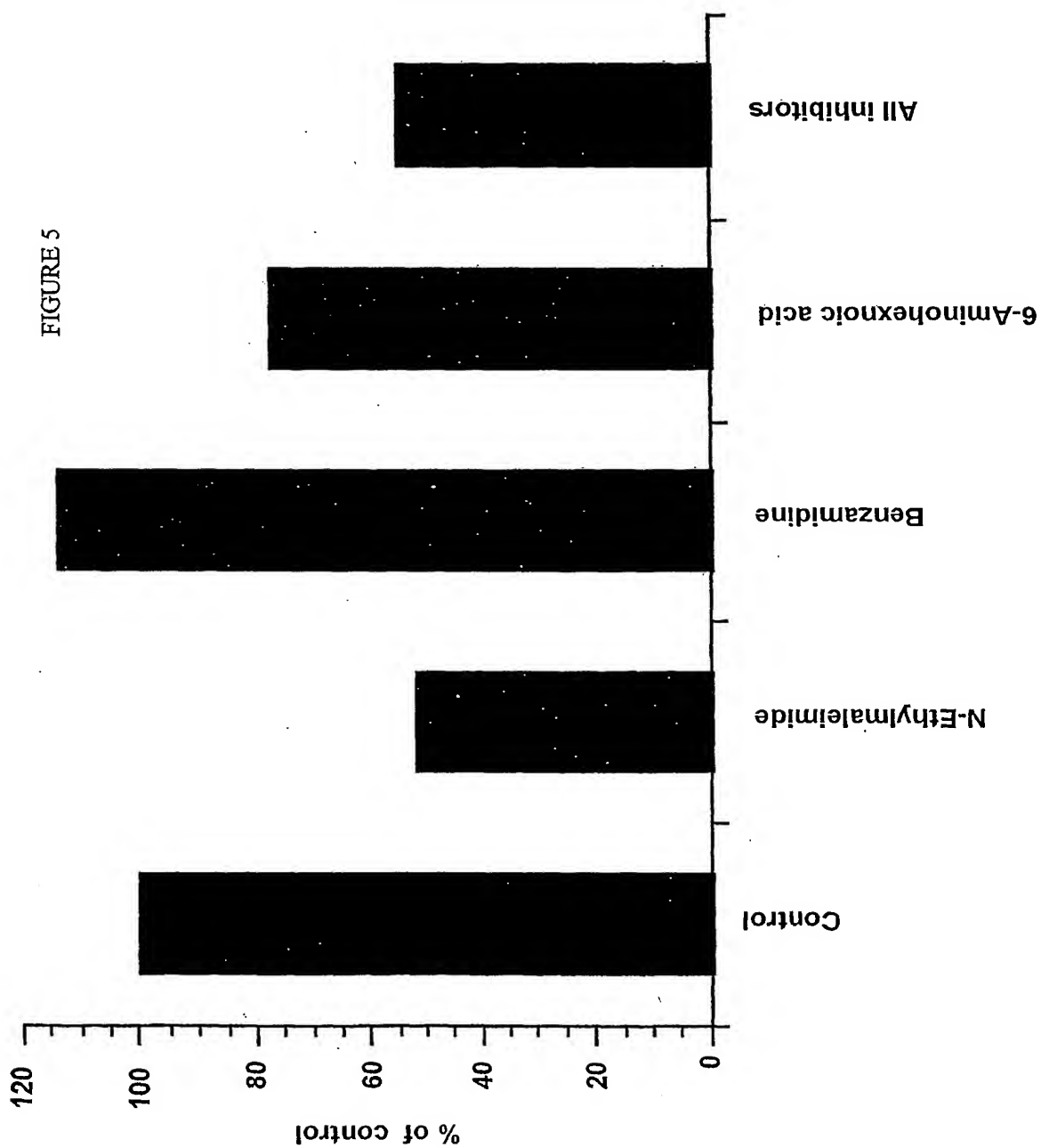
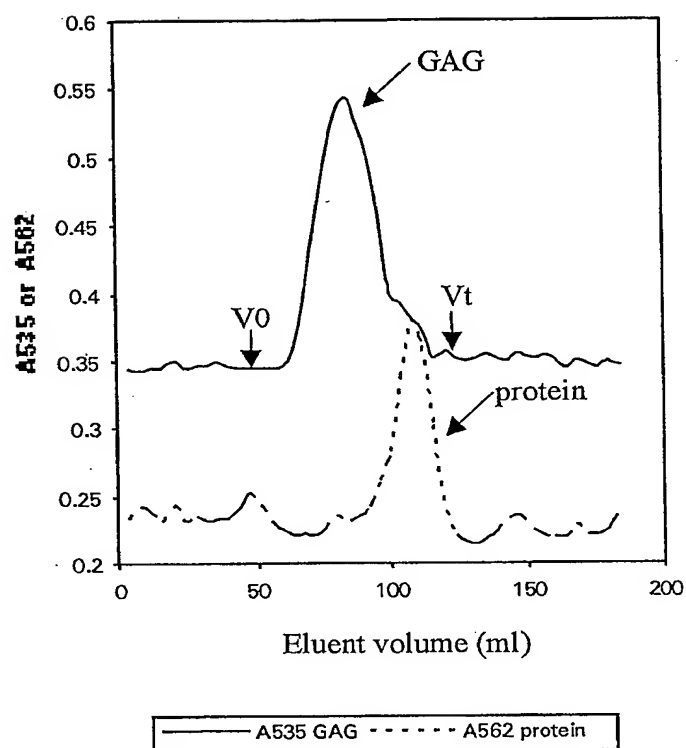
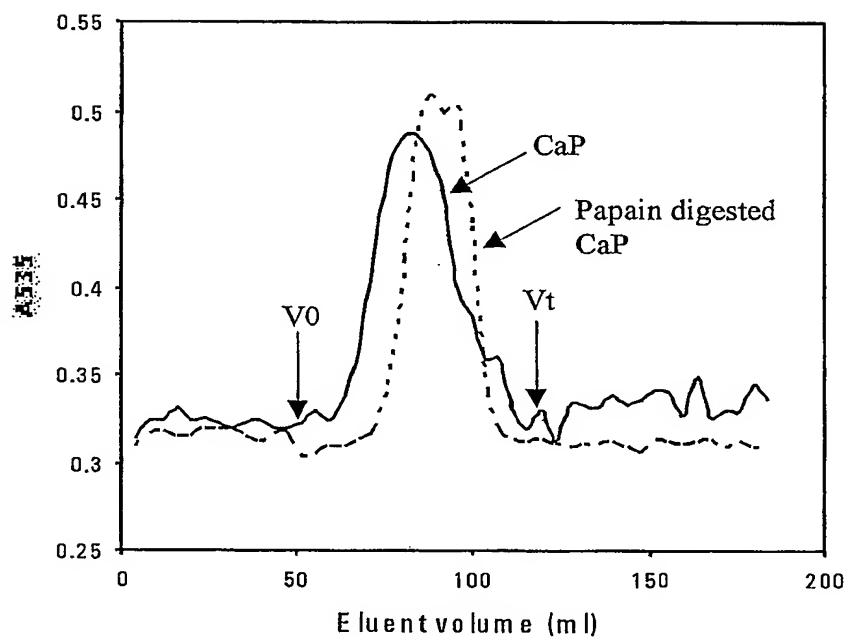
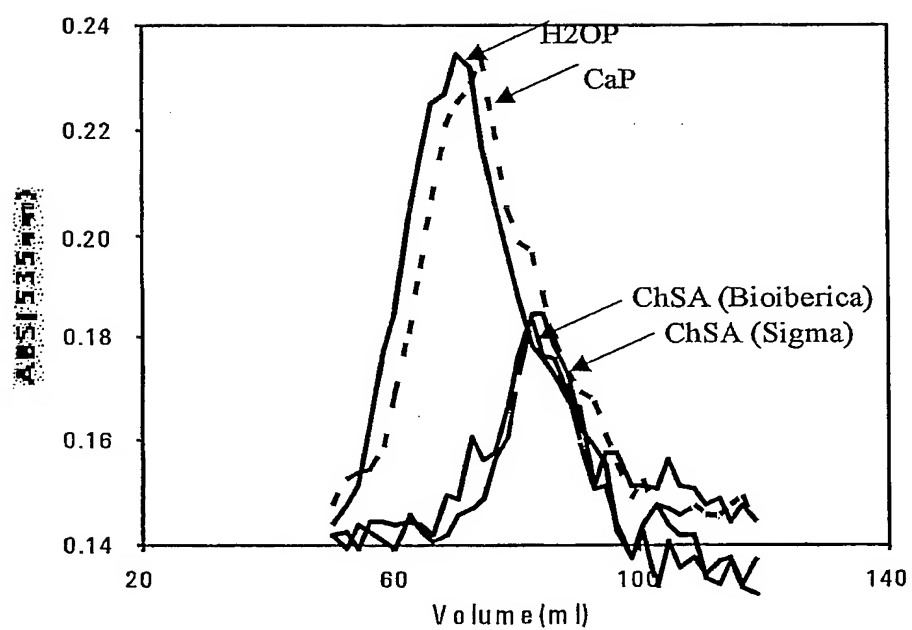


FIGURE 6

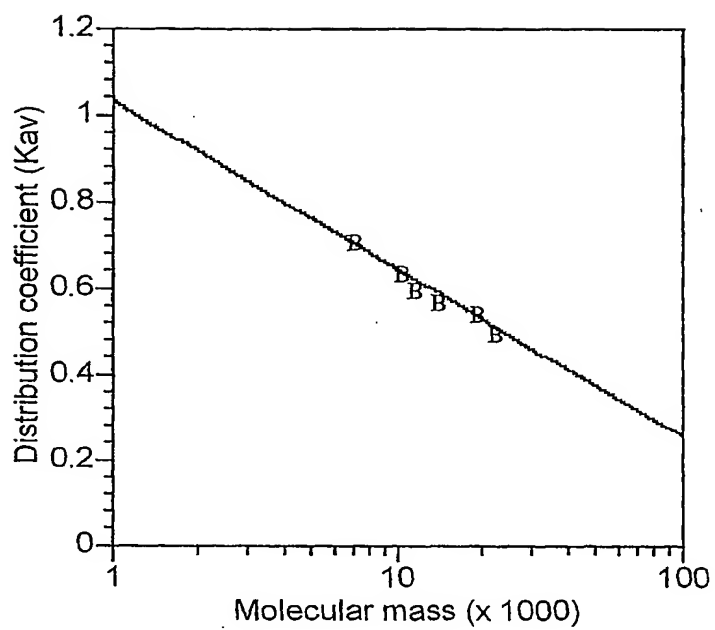


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FIGURE 7

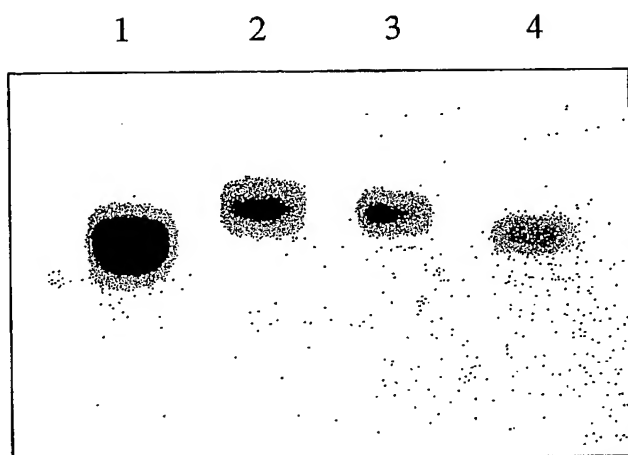


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FIGURE 8

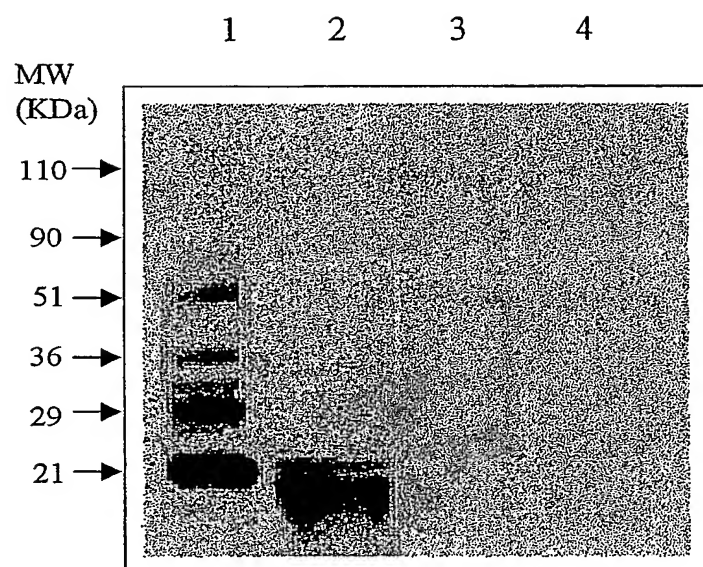
9/35
FIGURE 9

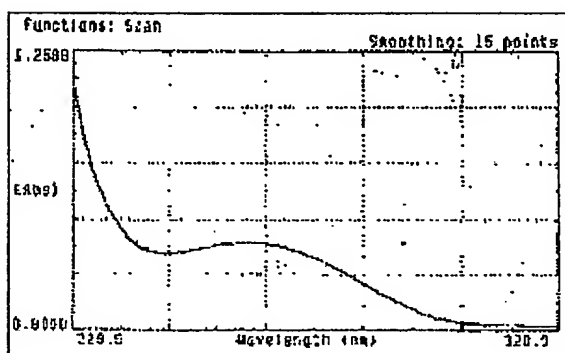
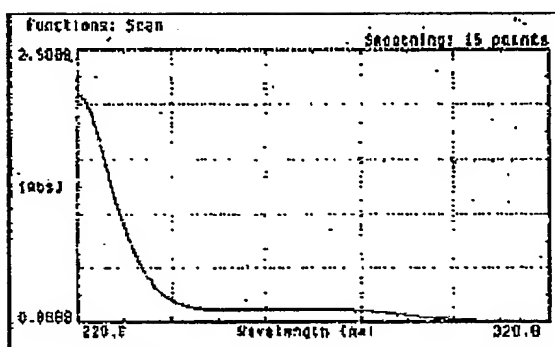
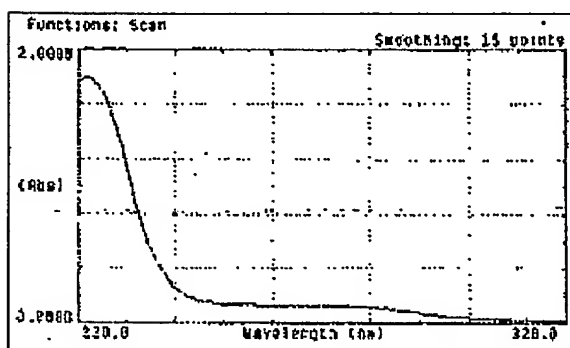
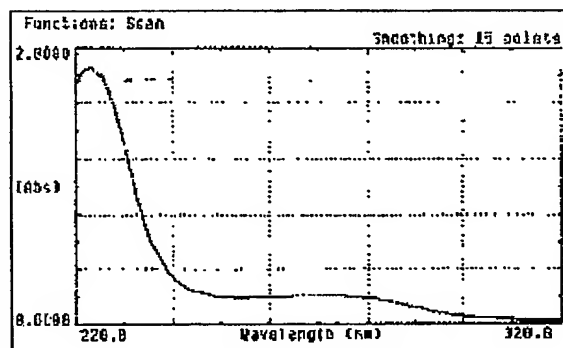


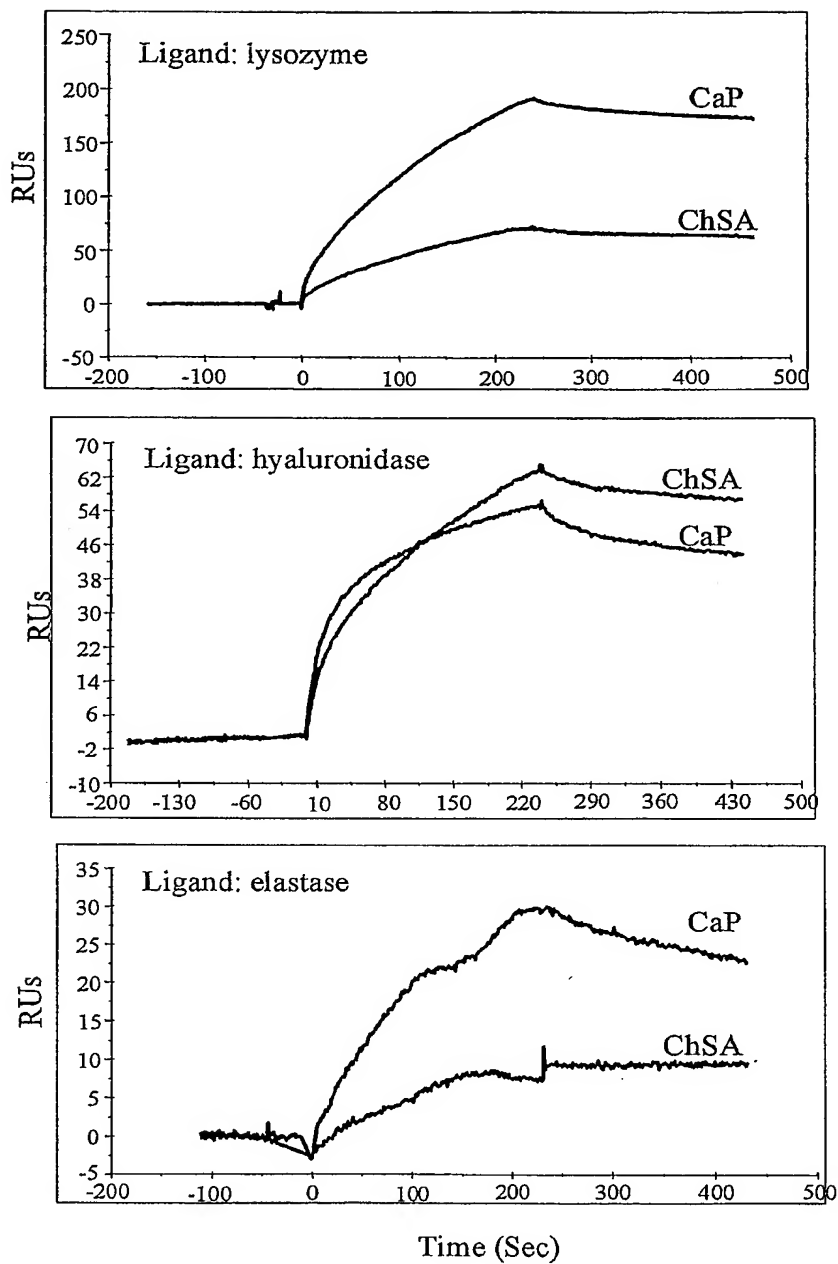
10/35
FIGURE 10

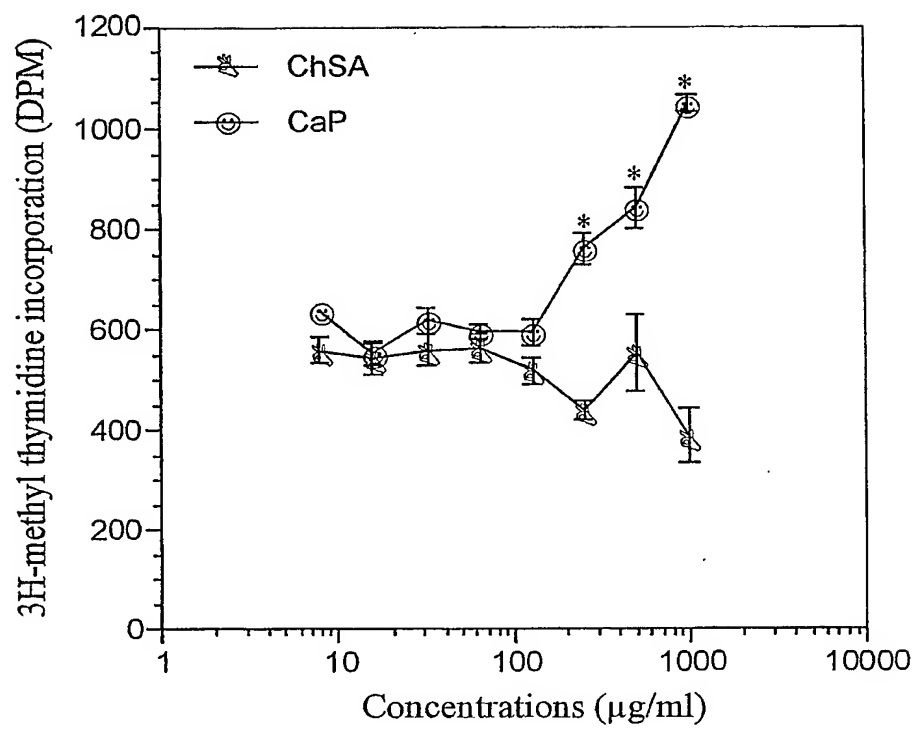


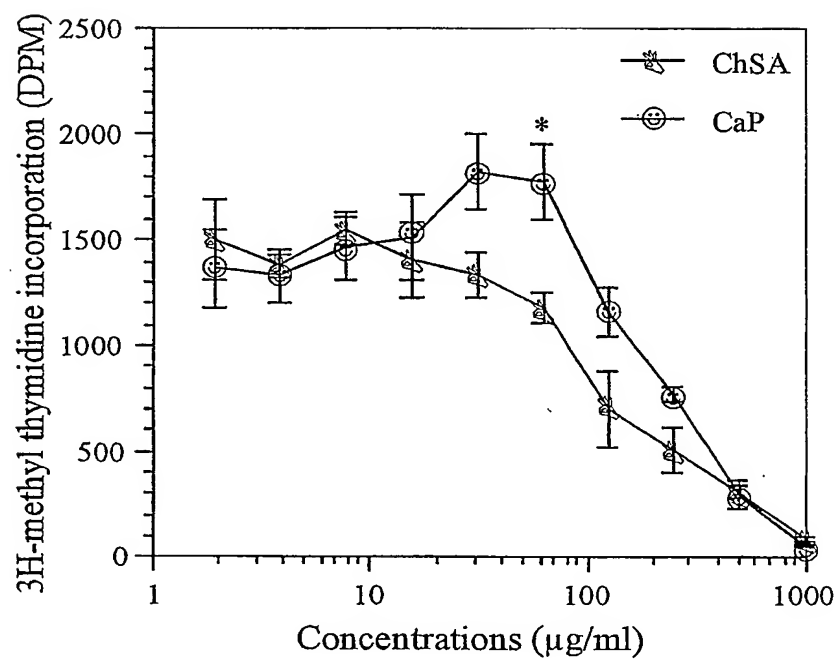
11/35
FIGURE 11

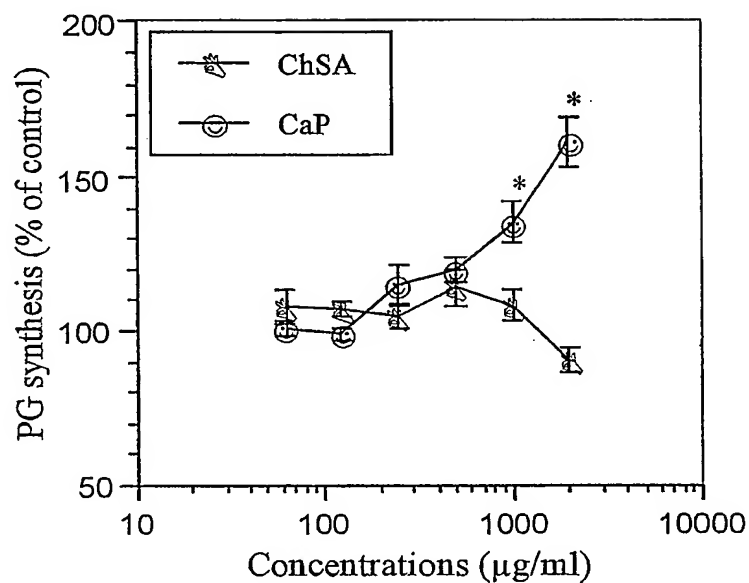
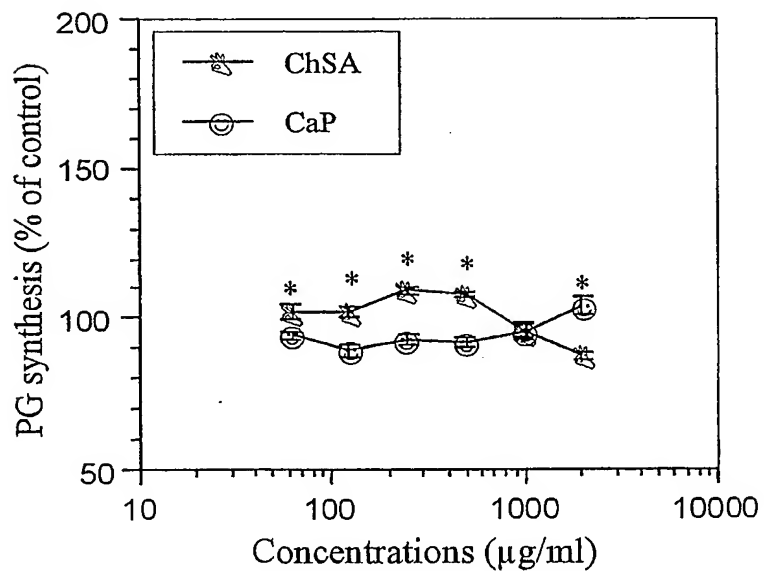


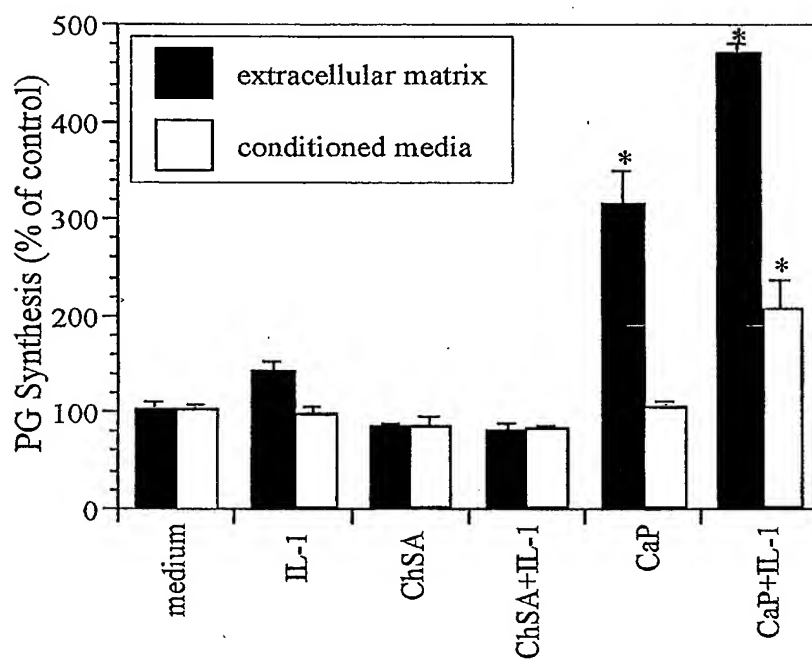
12/35
FIGURE 12ChSA ($A_{260}=0.39$; $A_{280}=0.21$)CaP ($A_{260}=0.12$; $A_{280}=0.11$)NaP ($A_{260}=0.13$; $A_{280}=0.12$)H2OP ($A_{260}=0.20$; $A_{280}=0.19$)

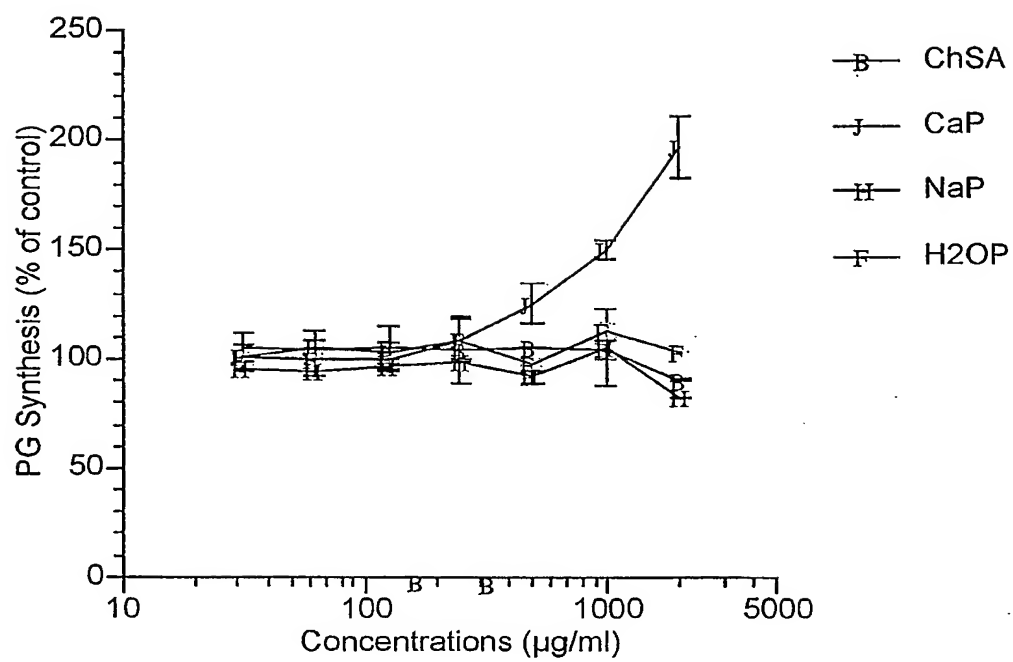
13/35
FIGURE 13

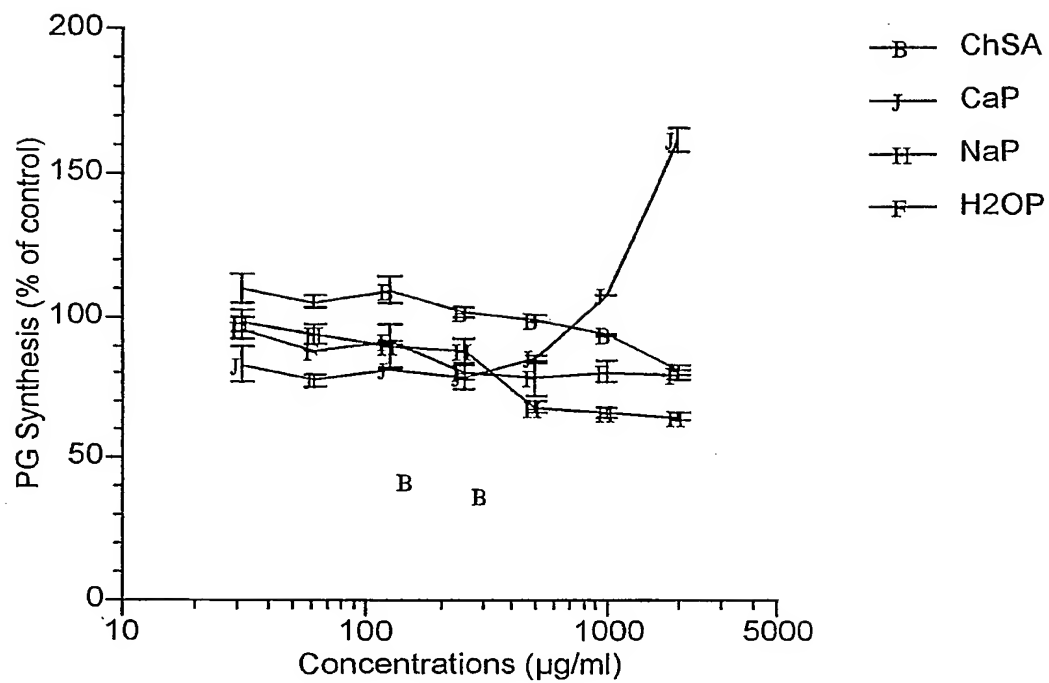
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FIGURE 14

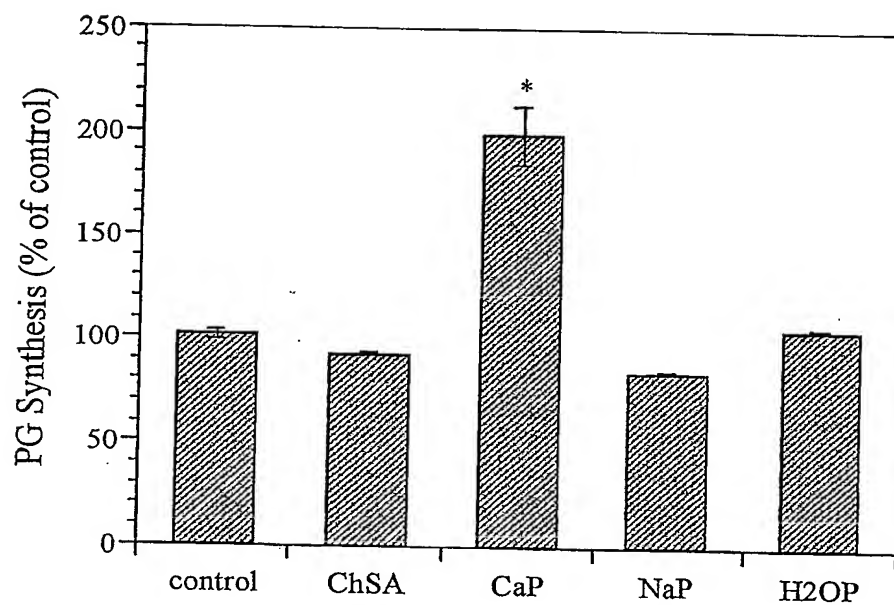
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FIGURE 15

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FIGURE 16**(A. extracellular matrix)****(B. media)**

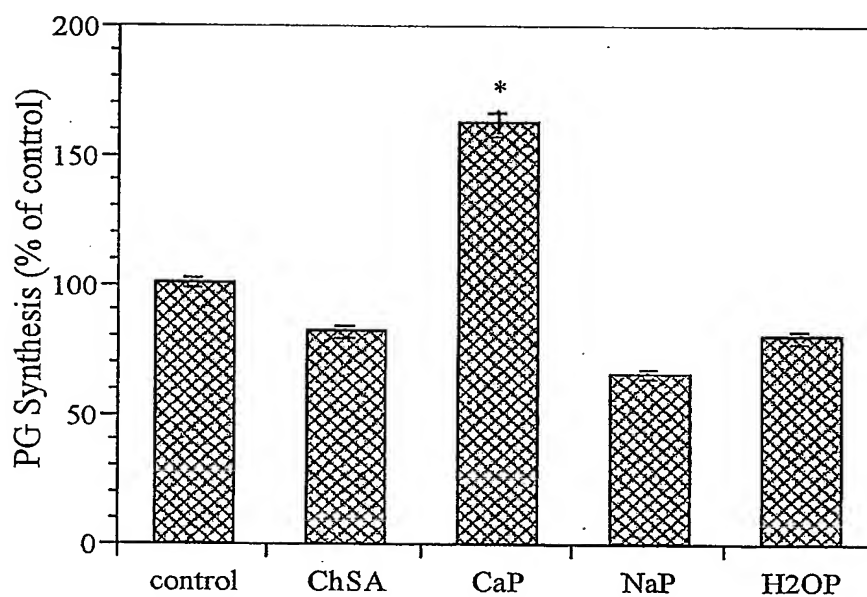
17/35
FIGURE 17

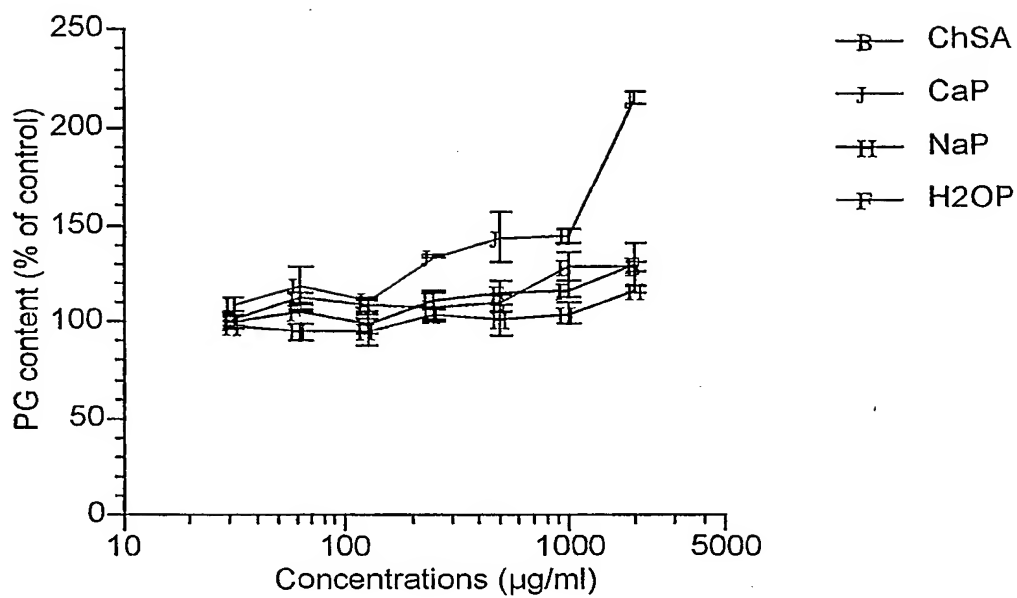
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FIGURE 18

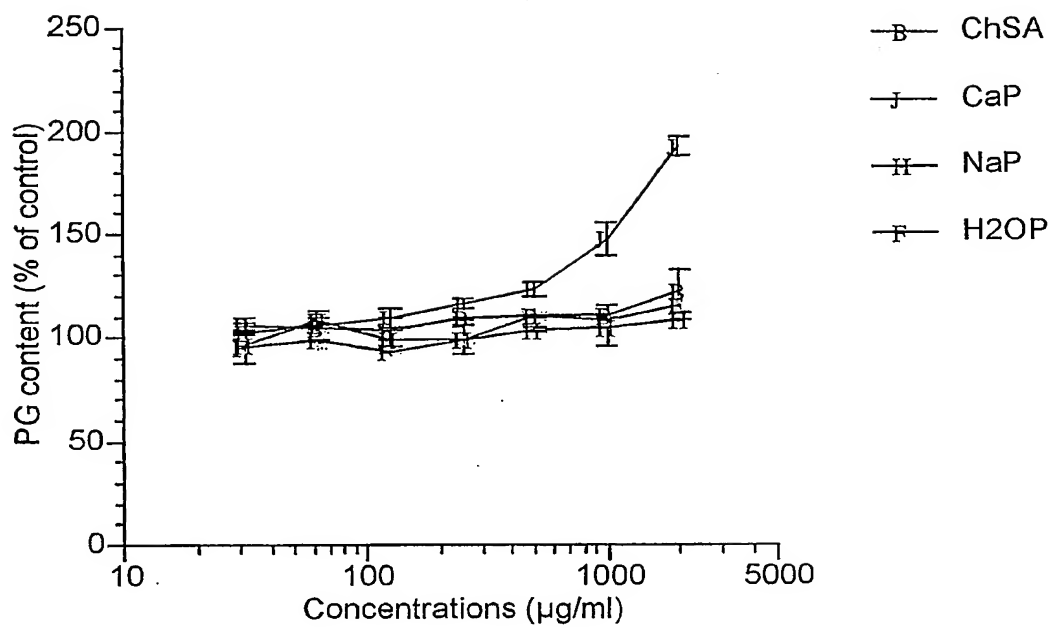
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FIGURE 19

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FIGURE 20

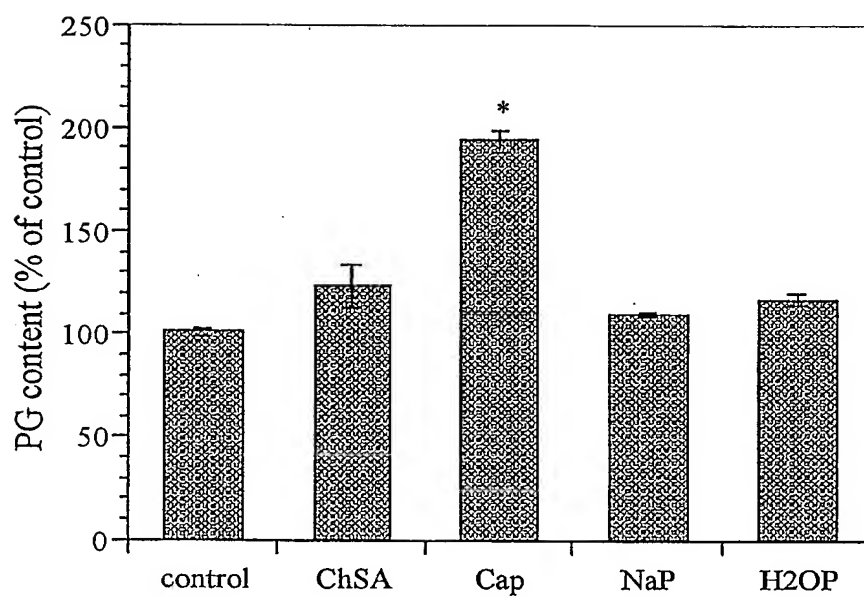
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FIGURE 21



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FIGURE 22

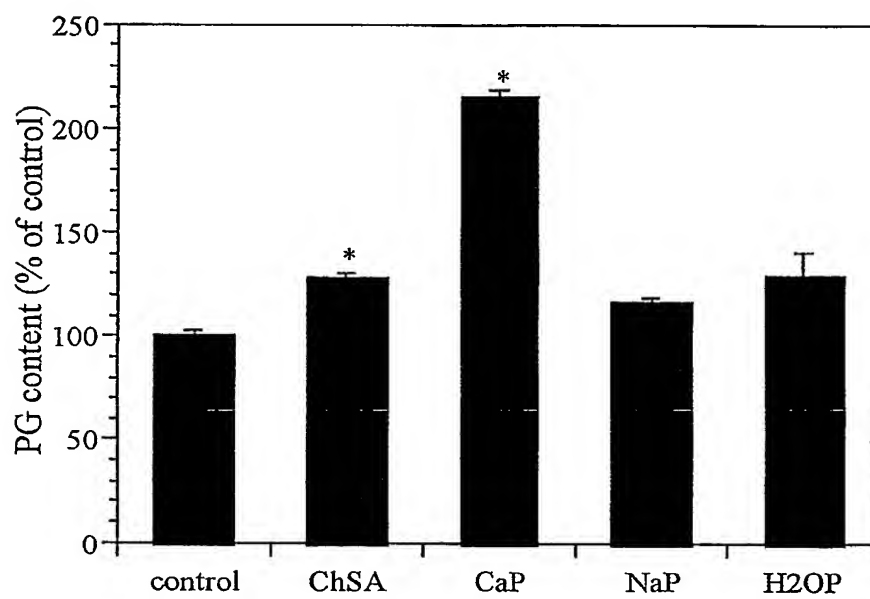
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FIGURE 23

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FIGURE 24



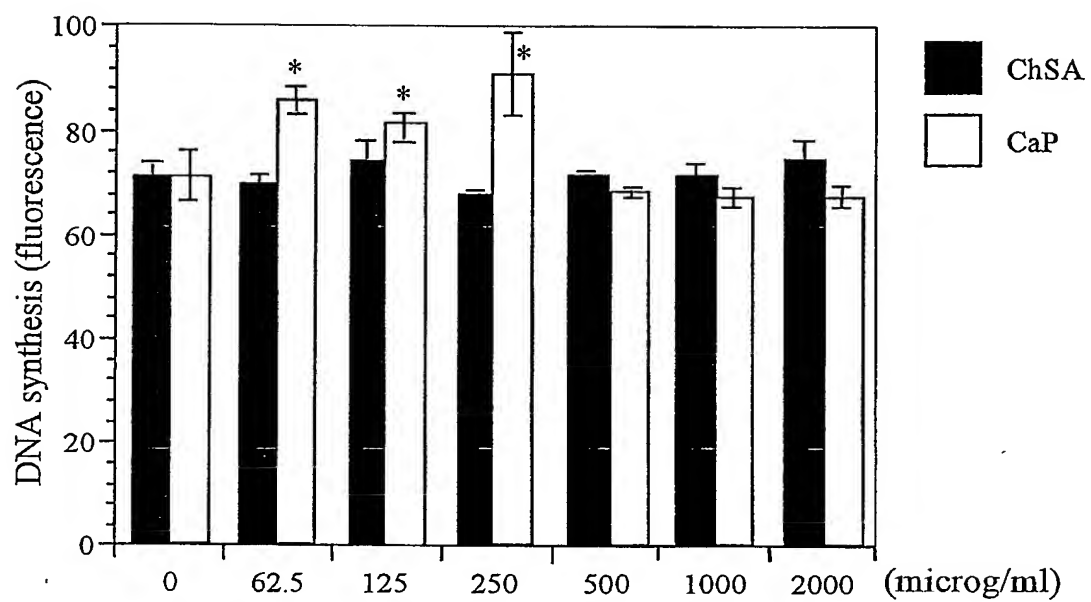
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FIGURE 25

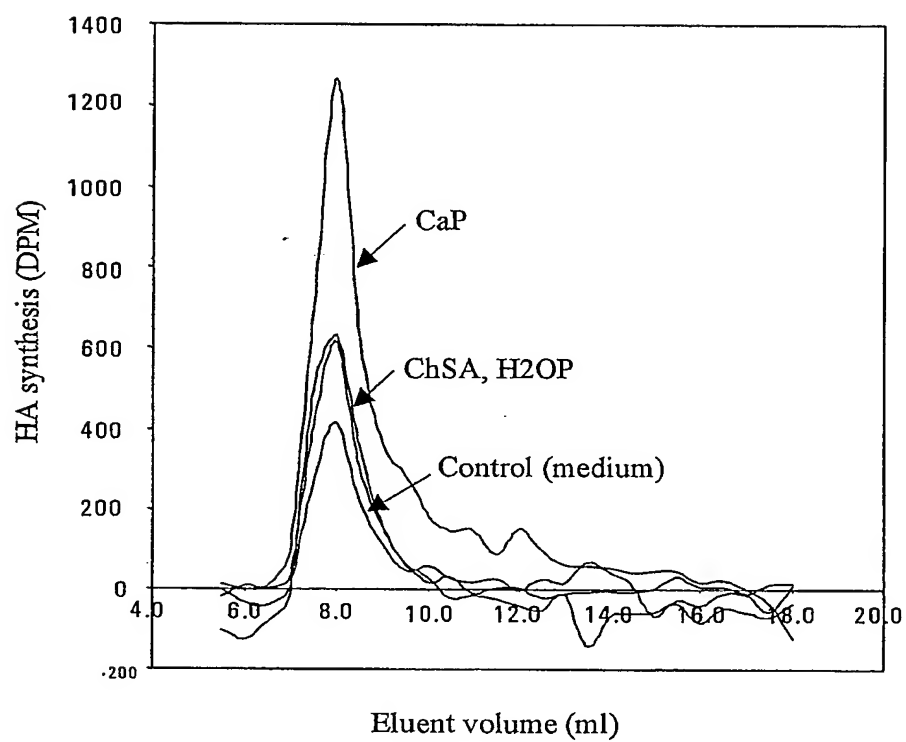


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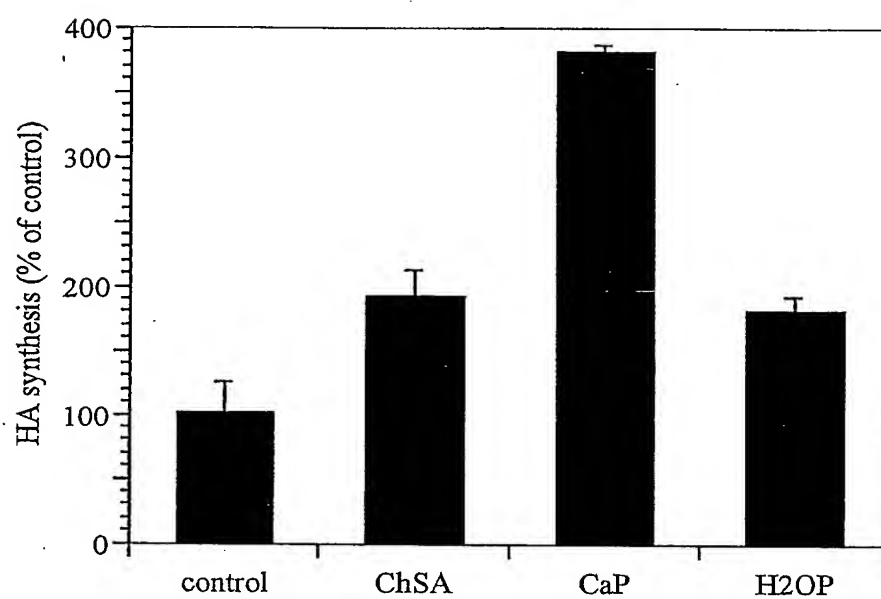
FIGURE 26



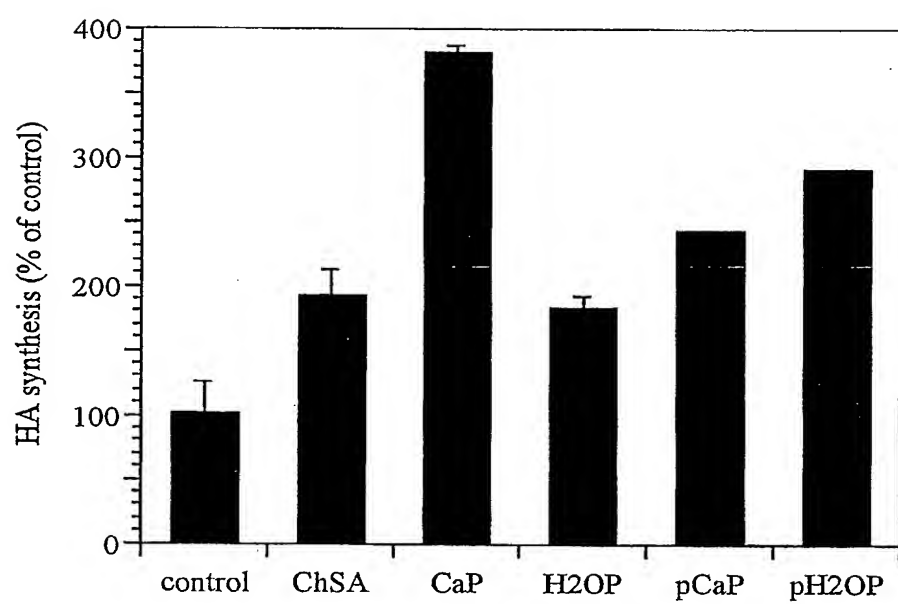
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FIGURE 27



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FIGURE 28

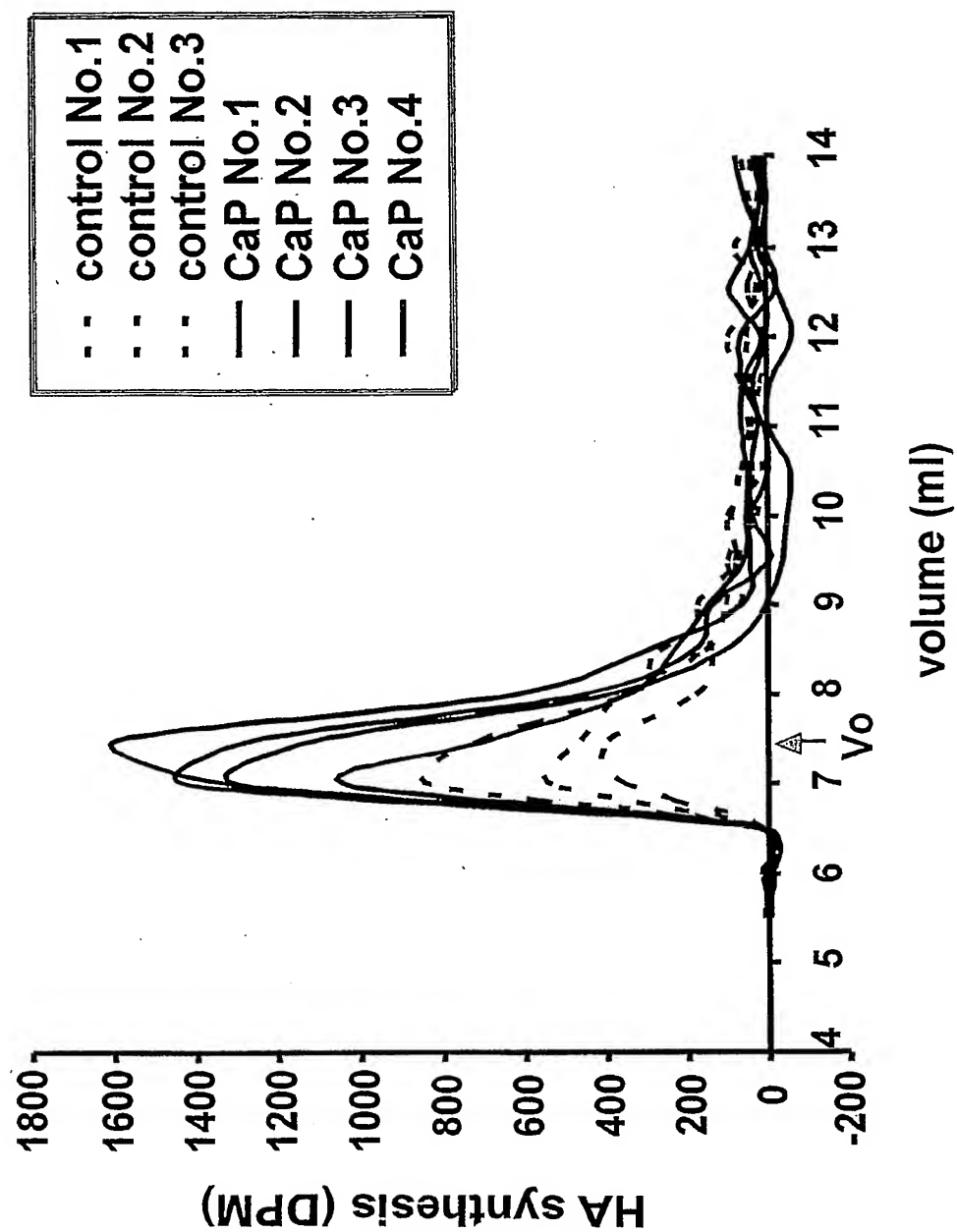


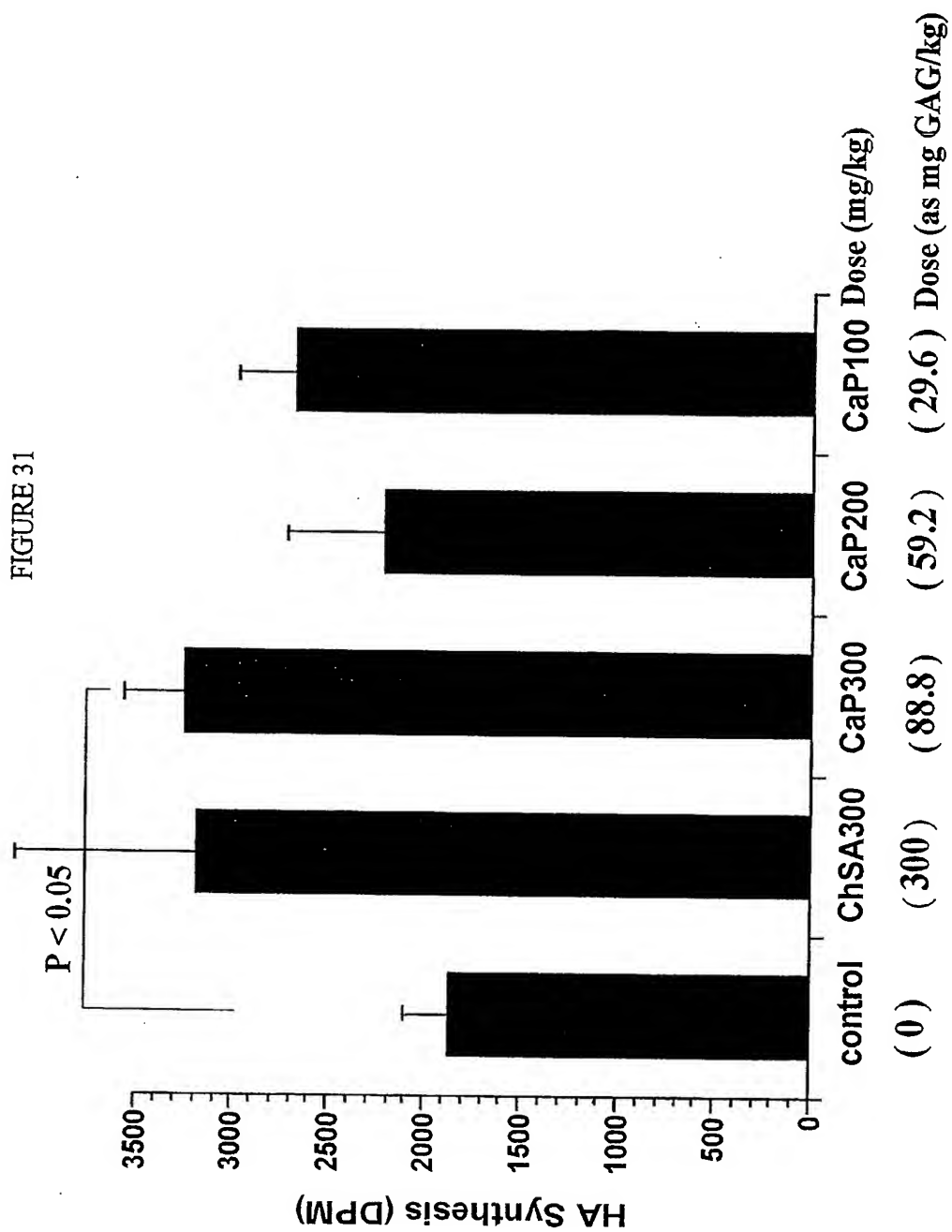
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FIGURE 29



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FIGURE 30





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FIGURE 32

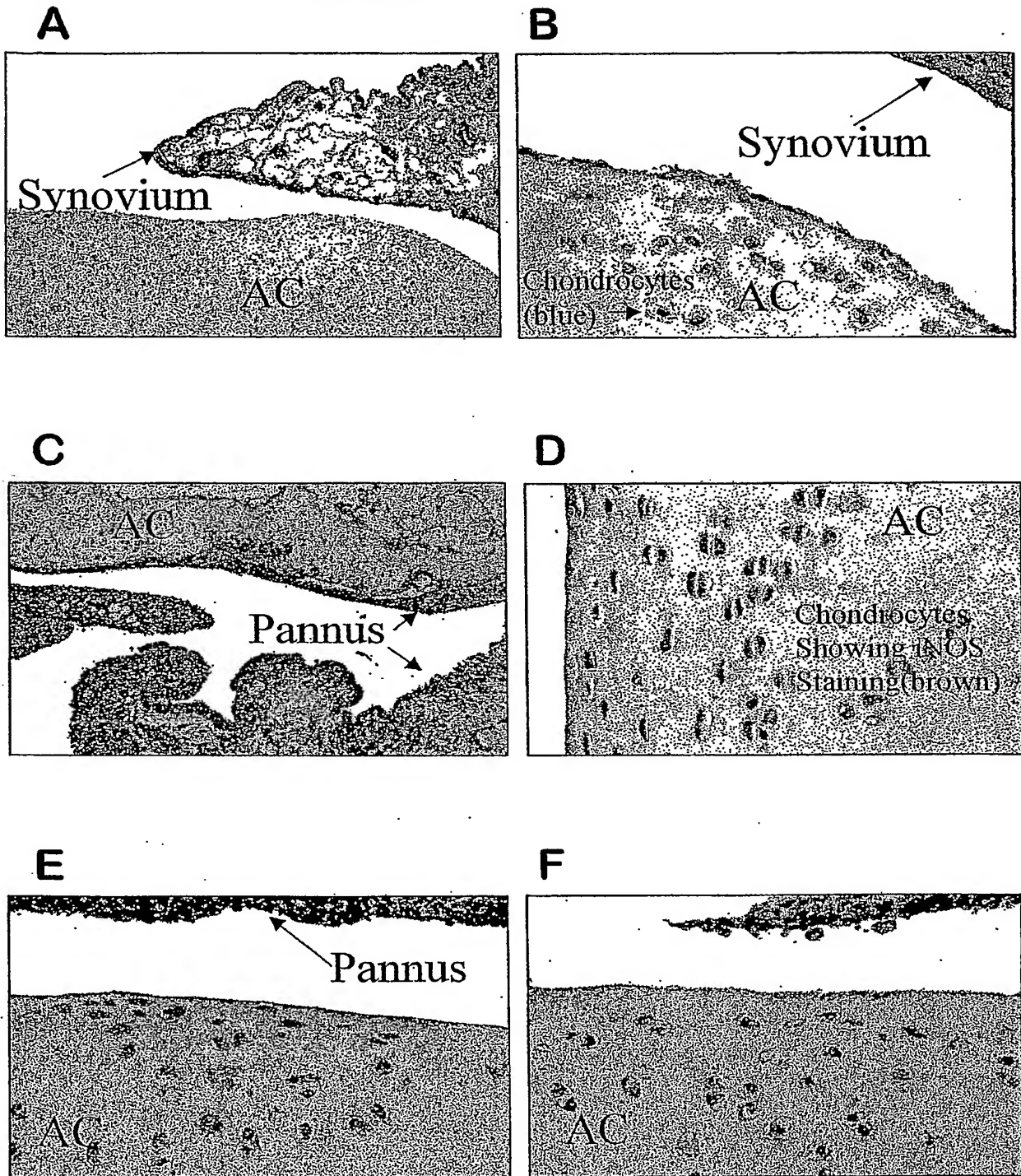


FIGURE 33

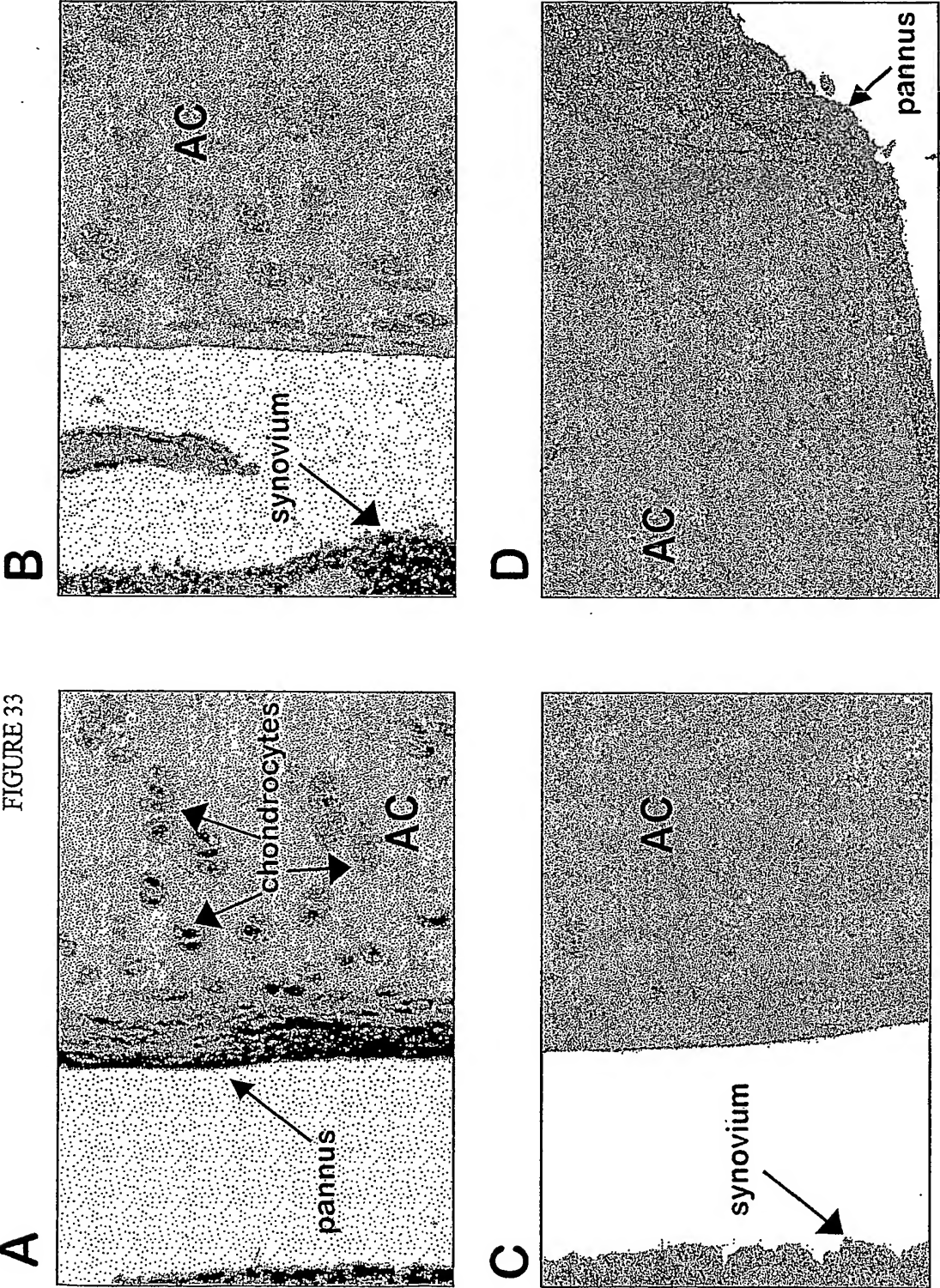


FIGURE 34

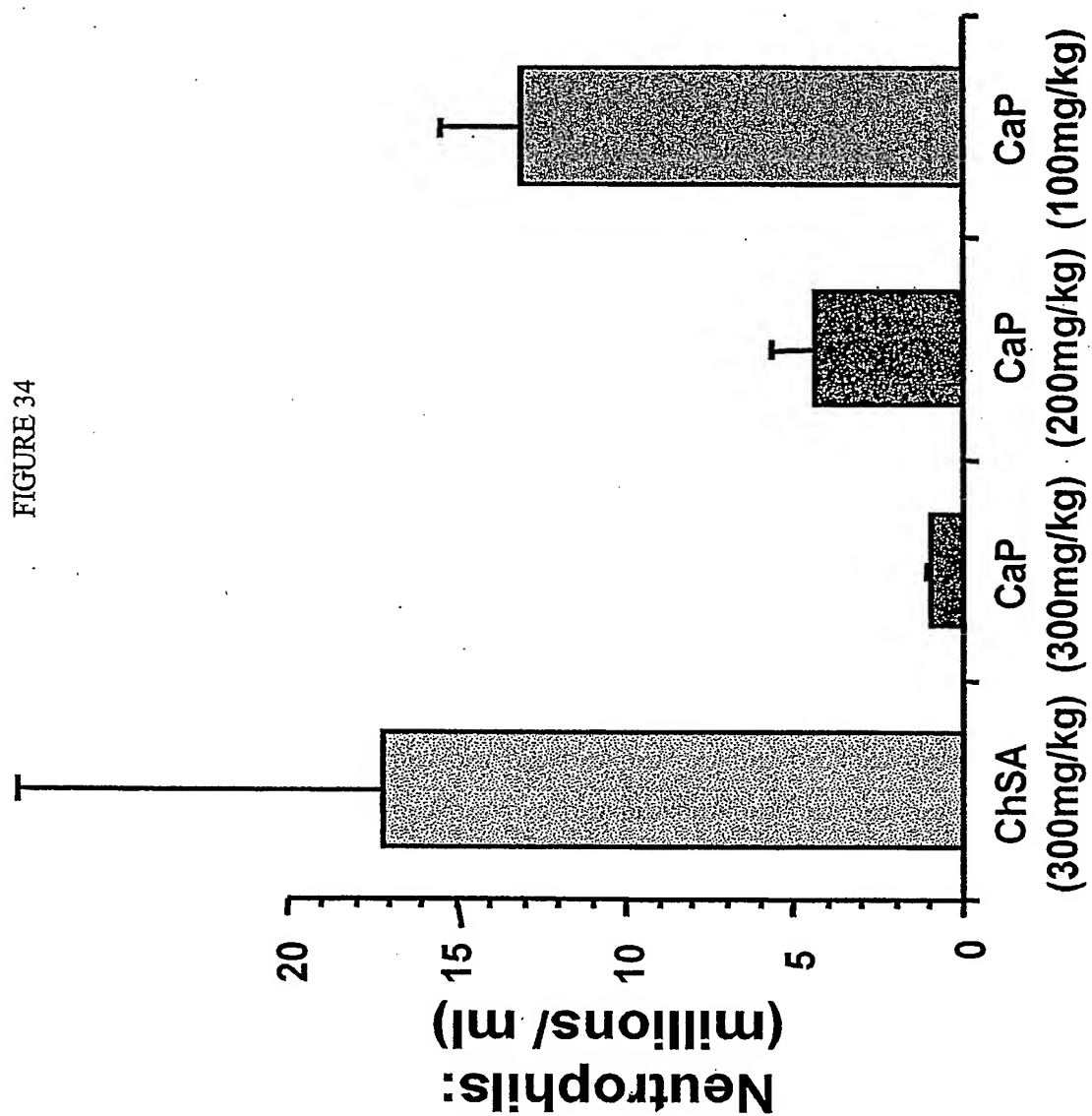
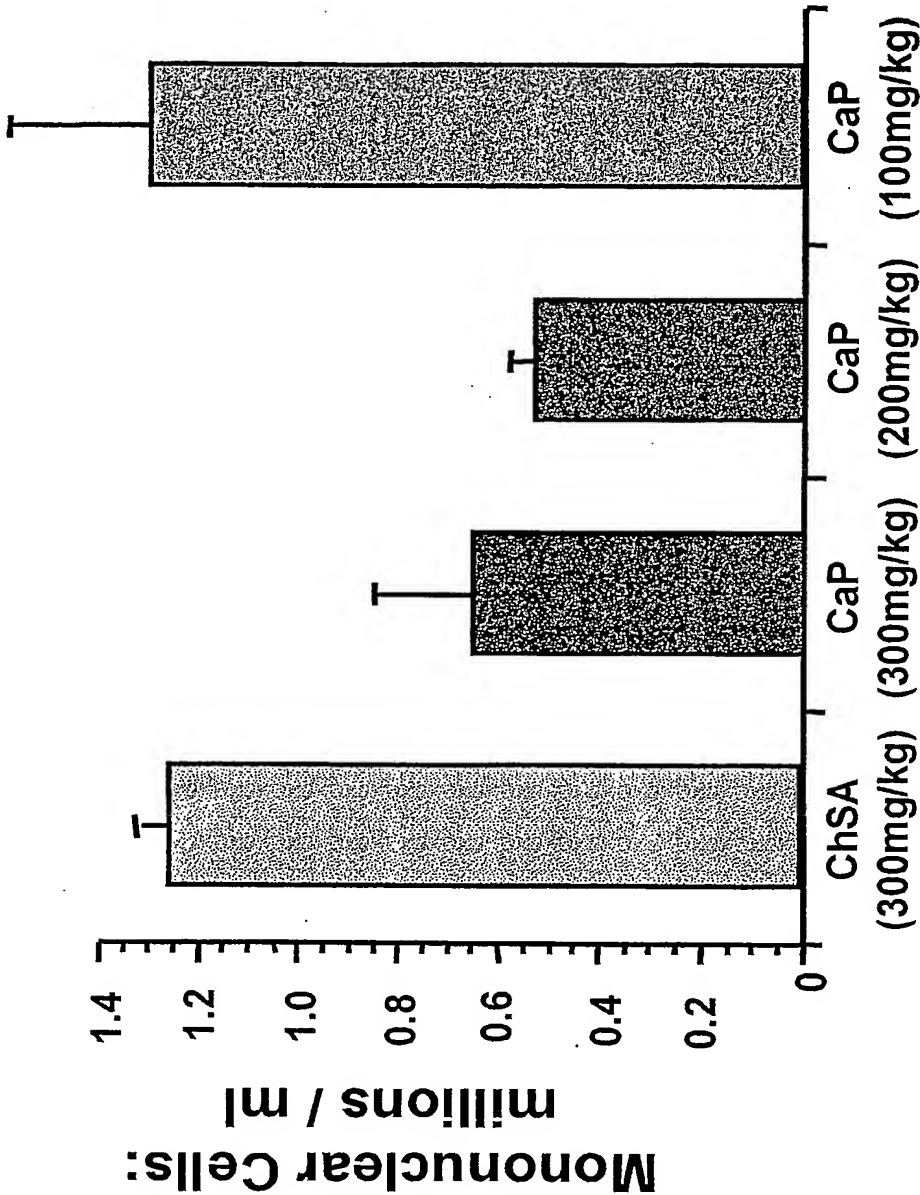


FIGURE 35



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00061

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: C08B 37/00; C07K 14/78; A61K 31/737, 31/726, 38/39; A61P 19/04, 29/00; C12S 3/02, 3/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN Files WPIDS, Medline, CA, Biosis: Keywords: glycosaminoglycan, GAG, Chondroitin sulfate, keratan sulfate, heparan sulfate, hyaluronic acid, peptacan, autolysis, extract, cartilage, connective, buffer, sodium acetate, calcium acetate, acetic acid and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Agricultural and Food Chemistry, Vol. 46, No. 2, 1998, Nakano et al, "Extraction of Glycosaminoglycan Peptide from Bovine Nasal Cartilage with 0.1 M Sodium Acetate", pages 772-778 Whole Document	1-15, 21-24, 28-32
X	Canadian Agricultural Engineering, Vol. 42, No. 4, 2000, Nakano et al, "An economical method to extract chondroitin sulphate-peptide from bovine nasal cartilage", pages 205-208 Whole Document	1-15, 21-24, 28-32
X	The Journal of Clinical Investigation, Vol. 51(9), 1972, Greenwald et al, "Human Cartilage Lysozyme", pages 2264-2270 See especially page 2265, col 1, page 2266 Table 1	1-13

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 2 April 2003	Date of mailing of the international search report 15 APR 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustrialia.gov.au Facsimile No. (02) 6285 3929	Authorized officer R.L. POOLEY Telephone No : (02) 6283 2242

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU03/00061

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages (Remove spaces when completed if the page is too long)	Relevant to claim No.
X	Biochemical Journal, Vol. 121(2), 1971, Brandt et al, "Heterogeneity of protein-polysaccharides of porcine articular cartilage. Sequential extraction of chondroitin sulfate proteins with iso-osmotic neutral sodium acetate", pages 261-270 See especially page 262	1-13
X	The Journal of Rheumatology, Vol. 27(2), 2000, Klein et al, "Immunomodulatory properties of Rumulon™, a Glycosaminoglycan peptide complex, in patients with Osteoarthritis: Activation of T Helper Cell Type 2 Cytokines and Antigen-Specific IgG4 Antibodies", pages 448-454 See abstract	11-16, 21-29
X	STN CA Online Abstract Accession No. 95:183447, Kosyagin, D.V, "Modification of a method for isolation of the proteoglycan fractions from cartilage" & Ukrainskii Biokhimicheskii Zhurnal, Vol.53(5), 1981, pages 90-92	1-13
X	International Congress series, Vol, 1223, 2001, Majima et al, "Effect of proteoglycan on experimental colitis", pages 221-224 See especially pages 222-223	1-13
X	BE 857389 A (MARBEX FINANCE LIMITED) 1 December 1977 See especially pages 2 & 4	13-15, 17, 22-25, 28,29
P,X	Poultry Science, Vol. 81 (7) 2002, Luo et al "Chicken Keel cartilage as a source of Chondroitin Sulfate", pages 1086-1089 See especially pages 1087 & 1089	1-16, 21-32
X	Journal of Biochemistry, Vol. 83(1), 1978, Tanaka, Kiichiro, "X-Ray Analysis of the Conformation of Chondroitin-4-Sulphate Calcium salt", pages 325-327 Whole Document	13,28
X	US 6162787 A (SORGENTE et al) 19 December 2000 See especially column 4	21, 30
P,A	US 2002/0068718 A (PIERCE) 6 June 2002	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU03/00061

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos : 12, 18, 19, 21, 27, 30
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 12, 18, 19, 21, 27 and 30 are directed to polypeptide salts and the use of polypeptides having indeterminate physical characteristics and chemical structures. Consequently for economic reasons, the search of these claims has been restricted to embodiments wherein these polypeptides are derived from the process of claim 1.
3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU03/00061

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member
		(To put a line under the citations tab to the first point on the next row and press F8)
BE	857389	NIL
US	6162787	NIL
US	2002/0068718	NIL
		END OF ANNEX

(To add more lines press TAB at end of last row, remove paragraph marker to join up 'END OF ANNEX' box)